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High Efficiency Reconstitution of a Phencyclidine/MK-801 Receptor Binding Site Solubilized from Rat Forebrain Membranes

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SUMMARY

Phencyclidine (PCP) receptors have been solubilized from rat forebrain membranes with the zwitterionic detergent 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate. Specific binding of the potent PCP receptor ligands [^{3}H]thienyl-phencyclidine (TCP) and [^{3}H]MK-801 was restored by incorporating extracted membrane protein into lipid vesicles prepared from a total brain lipid extract. A nearly quantitative recovery of solubilized receptor activity was achieved; this was dependent upon both the concentration of detergent used during membrane solubilization and the concentration of added lipid used during the reconstitution. The single, saturable, binding site measured for both [^{3}H]TCP and [^{3}H]MK-801 in solubilized and reconstituted preparations exhibited properties similar to those of the high affinity PCP binding site labeled by these ligands in brain membranes. The ability of ligands selective for this site (MK-801, TCP, and dexoxadrol) to competitively displace specific [^{3}H]TCP binding was retained after solubilization and reconstitution, although IC_{50} values measured for these ligands were shifted to higher concentrations. Levoxadrol and haloperidol were ineffective at displacing the radioligand binding in both membrane and vesicle preparations. The additive and dose-dependent ability of glutamate and glycine to enhance [^{3}H]TCP binding to the solubilized/reconstituted receptor further suggests that a direct interaction with the *N*-methyl-D-aspartate receptor/ion channel complex has been preserved in the vesicle preparations. The photoaffinity labeling of two polypeptides (M_r 98,000 and 59,000) by azido-[^{3}H]PCP was demonstrated in the vesicle preparations; this was largely prevented by competitive displacement of the radioligand with PCP before photolysis. These results establish both an essential lipid dependency and polypeptide composition for the high affinity, haloperidol-insensitive, PCP receptor in brain.

PCP and related arylcyclohexylamines are dissociative anesthetics used primarily as animal tranquilizers. They produce a number of psychotomimetic effects in humans [properties reviewed by Sonders *et al.* (1)], which are mediated via drug interaction at cell surface receptors located in the central nervous system. Both high (K_d = 10–20 nM) and low (K_d = 100–200 nM) affinity PCP binding sites have been measured in rat brain (2–5), but drug potencies in behavioral tests are correlative only with a high affinity interaction (6, 7).

Several high affinity PCP binding sites in brain can be further distinguished on the basis of differential drug potencies and anatomical distributions in the central nervous system (8–10). Ligand binding at these sites is blocked by the D-isomer of dioxodrol (dexoxadrol) but not by its corresponding L-isomer (levoxadrol) (4, 11). Drug interaction at σ^2 sites, initially identified on the basis of a selective benzomorphan action (13), can be competitively displaced by the neuroleptic drug haloperidol (14). A high affinity but haloperidol-insensitive PCP site also present in brain selectively binds both the thienyl-PCP analog TCP and the anticonvulsant MK-801 (9, 15).

Both TCP and MK-801 are noncompetitive antagonists of glutamate receptors of the NMDA subtype (16, 17) and act by blocking a nonselective cation channel gated by this receptor (18, 19). Ligand binding at this site results in selective channel inactivation in a use-dependent manner; this is facilitated by opening of the channel induced by NMDA receptor agonists (20). Drug binding at sites within this channel may provide a means for regulating cellular calcium homeostasis and ischemic

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[2] This was previously referred to as the σ-opiate receptor. Current nomenclature was described by Quirion *et al.* (12).

ABBREVIATIONS: PCP, 1-(1-phenylcyclohexyl)piperidine; TCP, *N*-[1-(2-thienyl)cyclohexyl]piperidine; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate; NMDA, *N*-methyl-D-aspartate; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

neuronal death, by blocking excitatory neurotransmission via the NMDA receptor/ion channel complex [NMDA receptor properties reviewed by Cotman *et al.* (21)].

Little is known about the membrane-associated structural determinants that dictate high affinity PCP binding to the site associated with the NMDA receptor. A lipid dependency similar to that shown for the nicotinic acetylcholine receptor/ion channel complex (22, 23) is likely, because ligand binding to PCP receptors is incompletely restored after their solubilization from membranes in detergent (24–26). Several laboratories have used a photoaffinity cross-linking approach in order to identify the brain membrane-associated polypeptides that bind PCP. Haring *et al.* (5) labeled five polypeptides with azido-[^{3}H]PCP, which ranged in size from M_r 90,000 to 33,000. The labeling of two polypeptides (M_r 90,000 and 33,000) was prevented by coincubating samples with PCP/σ ligands. The density of the M_r 90,000 polypeptide in various brain regions correlated with the localization of high affinity PCP binding sites (27), whereas the M_r 33,000 polypeptide was co-localized with low affinity sites. Sorensen and Blaustein (28) also demonstrated the covalent labeling of multiple brain membrane polypeptides with azido-[^{3}H]PCP; TCP displaced the radiolabel from M_r 95,000 and M_r 80,000 bands. In both studies, the unequivocal identification of receptor-associated polypeptides was hampered by the combined presence of high and low affinity PCP binding sites in the membrane preparations.

In this paper, we show that a highly efficient recovery of ligand binding activity can be achieved by reconstituting a CHAPS-solubilized PCP binding site into lipid vesicles under conditions that allow its reassociation with activating lipids. Pharmacological characterization of the reconstituted receptor showed that properties similar to those of the high affinity, haloperidol-insensitive, PCP receptor site in brain were preserved. The data further suggest that the reconstituted receptor site is associated with the NMDA receptor/ion channel complex. These partially fractionated preparations possessed levels of PCP receptor binding activity that were considerably higher than those found in membranes. Only two of the polypeptides labeled previously in membranes by azido-[^{3}H]PCP were similarly identified in these solubilized/reconstituted samples.

Experimental Procedures

Materials. [^{3}H]TCP (48.9 Ci/mmol) and [^{3}H]MK-801 (29.4 Ci/mmol) were purchased from New England Nuclear. Azido-[^{3}H]PCP (50.0 Ci/mmol) was purchased from Israel Nuclear Center (Negev, Israel). CHAPS was purchased from Calbiochem. Haloperidol, L-glutamate, and the Folch total brain lipid extract (type VII) used in reconstitution studies were purchased from Sigma. The high intensity Spectroline UV lamp used in photoaffinity labeling experiments, capable of an emission maximum at 254 nm of 650 μW/cm^2 (measured at a distance of 15 cm), was purchased from Fisher. PCP and TCP were generous gifts of Dr. M. Sokolovsky (Tel Aviv University, Tel Aviv, Israel).

Membrane preparation. Membranes were prepared from adult male Sprague-Dawley rat forebrain (whole brain minus cerebellum and brainstem) essentially as described by Haring *et al.* (5). Well washed membranes were used to measure the glutamate and glycine enhancement of [^{3}H]TCP binding; these were prepared as described by Reynolds *et al.* (29). Briefly, rat forebrains were homogenized with a Brinkman Polytron homogenizer, in 30 volumes of 20 mM HEPES buffer, pH 7.4, containing 1 mM EDTA, and were collected by centrifugation at 48,000 × *g* for 10 min. This pellet was processed four times at 4° in this manner and an additional three times in the absence of EDTA. These membranes were resuspended in 0.32 M sucrose and stored at −70°.

Membrane solubilization. Membranes were detergent solubilized essentially as described by Scheideler and Zukin (30). High speed P_2 membranes, or washed synaptosomal membranes, were homogenized in buffer A (25 mM Tris·HCl buffer, pH 7.4, containing 0.1 M NaCl) containing 20% (w/v) glycerol and mixed with an equal volume of buffer A containing CHAPS, to yield a final membrane protein concentration of 10 mg/ml. After gentle shaking for 1 hr at 4°, the samples were centrifuged at 100,000 × *g* for 25 min in a Beckman model TL-100 ultracentrifuge, in order to remove insoluble material. The supernatant could then be frozen at −70° for at least 1 week with no loss of ligand-binding activity.

Receptor reconstitution. The conditions used to incorporate the soluble receptor into lipid vesicles are essentially the same as described by Scheideler and Zukin (30). Briefly, except where noted the lipid content of soluble receptor samples, containing 3 mg of membrane protein (and <25 mg of CHAPS), was enhanced by mixing for 1 hr at 4° with 6 mg of lipid vesicles prepared by direct probe sonication of a brain lipid extract. The vesicles that spontaneously formed after dilution of these samples 60-fold into ice-cold 25 mM potassium phosphate buffer, pH 7.4, were then collected by centrifugation for 5 hr at 100,000 × *g*. Pellets were rehomogenized in this same buffer and frozen at −70°. The ligand-binding activity of these preparations was stable for at least 4 days.

Assay of specific ligand binding. Membranes (100–150 μg of protein), soluble membrane extracts (100–150 μg of protein), or vesicle preparations (10 μg of protein) were incubated for 1 hr at 25°, in a total volume of 200 μl containing 5 mM potassium phosphate buffer, pH 7.4, and either [^{3}H]TCP or [^{3}H]MK-801; nonspecific binding was determined by also including 0.1 mM unlabeled PCP or MK-801, respectively. No change in radioligand occurred when glutamate and glycine were added to the assays unless well washed membranes were used in the assay or as a source of tissue during receptor solubilization. This result likely reflects the presence of high endogenous amino acid concentrations in the membrane preparations. Therefore, glutamate and glycine were not routinely added to the assays to potentiate radioligand binding. The activity of soluble membrane extracts (100–150 μg of protein) was also determined in this assay system; the detergent concentration of the samples was first diluted 10-fold, to avoid interference in the assay. Free and bound ligand were separated by vacuum filtration, on Whatman GF/B filters that had been treated with 0.1% polyethyleneimine (w/v), using a Millipore filtration manifold. Filters were added to vials containing 5 ml of Aquasol II (New England Nuclear), and bound radioactivity was measured in a Packard Tricarb 4550 scintillation counter. Results were expressed as averages of triplicate data. Sample points typically deviated from this average by <20%.

Data analysis. Data from radioligand binding experiments were evaluated using a weighted, nonlinear, least squares, curve-fitting program (LIGAND-PC) obtained from Dr. P. Munson at the National Institutes of Health (Bethesda, MD). This analysis yields values for the proportion of high and low affinity sites as well as the binding constants for these sites (31). Values of K_d and B_{max} are described in the text for each site; these represent the mean ± standard error of three or four experiments. Scatchard plots of B/F versus B are also shown for individual experiments in Figs. 1 and 4. B is the specific radioligand binding (pmol/mg) and F is the free concentration of radioligand (nM).

Photoaffinity labeling. Labeling was performed essentially as described previously by Haring *et al.* (5), with the following differences. Vesicle preparations (50 μg) were mixed with 20 nM azido-[^{3}H]PCP in 1 ml of 5 mM potassium phosphate buffer, pH 7.4, and then incubated in the dark for 1 hr at 25° before photolysis. A relative excess of PCP was included for determinations of nonspecific labeling. After protein separation by SDS-polyacrylamide gel electrophoresis, gels were fixed, stained, treated with ENHANCE (New England Nuclear), and dried.

Radiolabeled bands were identified on Kodak XAR-5 film. Films were exposed for 2 weeks at −70°, using regular intensifying screens.

Protein assay. Protein was estimated by the method of Lowry *et al.* (32), using bovine serum albumin as the standard. Membrane and reconstituted vesicle samples were assayed in the presence of added SDS, as described by Peterson (33).

Results

Specific radioligand binding to the PCP receptor in membranes. The potent and selective PCP receptor ligands [^{3}H]TCP and [^{3}H]MK-801 were used to measure specific binding to rat forebrain membranes. Saturable [^{3}H]TCP binding was achieved only at high ligand concentrations, due to the combined presence of high affinity (K_H = 16 ± 6 nM; B_H = 0.97 ± 0.2 pmol/mg) and low affinity (K_L = 59 ± 16 nM; B_L = 0.93 ± 0.28 pmol/mg) binding sites (Fig. 1A). Only a high affinity binding site interaction (K_d = 3.0 ± 0.9 nM; B_{max} = 0.7 ± 0.02 pmol/mg) was measured when the selective PCP receptor ligand [^{3}H]MK-801 was used in the assays (Fig. 1B). We further characterized this high affinity binding interaction by measuring the ability of receptor-selective ligands to displace [^{3}H]TCP binding from the brain membranes. These data are shown in Fig. 1C and summarized in Table 1. The ability of dexoxadrol, but not its behaviorally inactive L-isomer levoxadrol, to effectively compete with [^{3}H]TCP for specific binding was consistent with the 100–200-fold difference in their reported efficacies at PCP binding sites (4, 11). The specific radioligand binding did not result from a σ receptor interaction; haloperidol (1 μM) displaced only 25% of the total specific [^{3}H]TCP binding and was only marginally more effective when a 10-fold higher concentration was used. A selective PCP receptor interaction was confirmed by demonstrating that low concentrations of MK-801 competively displaced all of the specific [^{3}H]TCP binding. In addition, glutamate significantly increased the radioligand binding in washed membrane preparations; this effect was further enhanced by the coaddition of glycine (Table 2). Taken together, these results are consistent with the high affinity binding of both [^{3}H]TCP and [^{3}H]MK-801 at a site in membranes previously shown to be associated with the NMDA-subtype of glutamate receptor.

Solubilization and reconstitution of the PCP receptor. Rat forebrain (P_2) membranes were solubilized with increasing concentrations of the zwitterionic detergent CHAPS, and the extracts were reconstituted into lipid vesicles. Measurements of soluble protein in the membrane extracts and recoveries of specific radioligand binding activity are shown in Fig. 2. Solu-

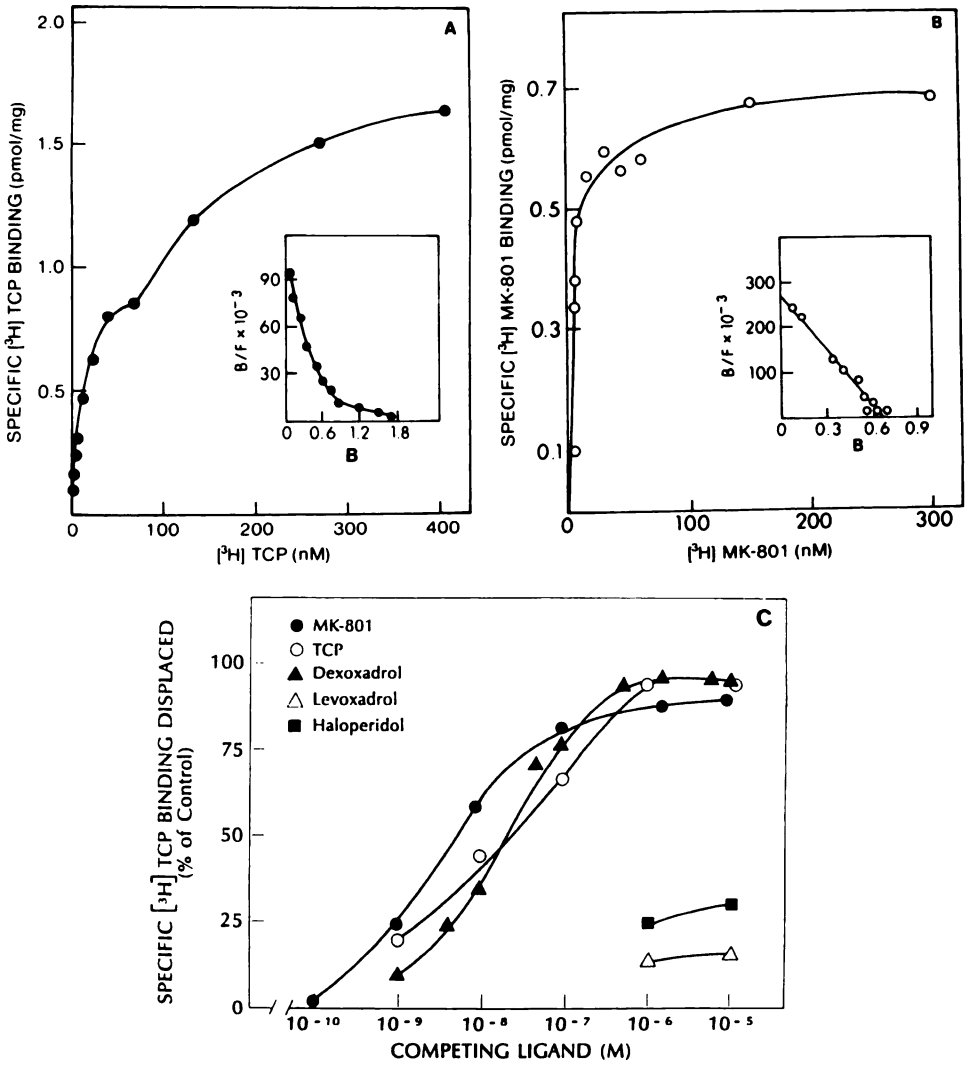

Fig. 1. Characterization of the PCP receptor in rat brain membranes. A, Specific [^{3}H]TCP binding. *Inset*, Scatchard plot of these data. B, Specific [^{3}H]MK-801 binding. *Inset*, Scatchard analysis of these data. C, The displacement of [^{3}H]TCP from membranes by MK-801 (●), TCP (○), dexoxadrol (▲), levoxadrol (△), and haloperidol (■) is presented as the fractional displacement of the total specific binding measured for 10 nM [^{3}H]TCP. The membrane preparation and assay methodology is described in Experimental Procedures. The data are representative of triplicate experiments.

TABLE 1

Competitive displacement of specific [^{3}H]TCP binding

Membrane preparation and solubilization with 10 mM CHAPS, reconstitution of membrane extracts, and assay of specific ligand binding are described in Experimental Procedures. The drug concentration reducing specific [^{3}H]TCP binding by 50% (IC_{50}) was determined experimentally in the presence of a [^{3}H]TCP concentration of 10 nM. IC_{50} values are expressed as the mean ± standard error of two to four experiments.

Ligand	IC_{50}	
	Membrane (P_2) preparations	Solubilized/reconstituted receptor
	nM	
MK-801	4 ± 1	360 ± 130
TCP	20 ± 5	92 ± 30
Dexoxadrol	18 ± 3	260 ± 100
Levoxadrol	>10,000[a]	>10,000
Haloperidol	>10,000	>10,000

[a] Complete displacement of [^{3}H]TCP binding was not achieved at the highest concentrations of levoxadrol and haloperidol used.

TABLE 2

Enhancement of specific [^{3}H]TCP binding by glutamate and glycine

Membrane preparation and solubilization with 10 mM CHAPS, reconstitution of membrane extracts, and assay of specific ligand binding are described in Experimental Procedures. Results are expressed as the mean ± standard error of three experiments. Measurements of specific binding were performed using a [^{3}H]TCP concentration of 10 nM.

Assay conditions	Specific [^{3}H]TCP binding	
	pmol/mg	*% of control*
Washed membranes	0.30 ± 0.02	100
+ 10 μM Glutamate	0.38 ± 0.03	126
+ 10 μM Glutamate + 30 μM glycine	0.49 ± 0.03	163
Solubilized/reconstituted receptor	1.59 ± 0.24	100
+ 1 μM Glutamate	2.40 ± 0.54	151
+ 10 μM Glutamate	2.88 ± 0.57	181
+ 100 μM Glutamate	3.94 ± 0.51	248
+ 1 μM Glutamate + 1 μM glycine	3.66 ± 0.87	230
+ 1 μM Glutamate + 30 μM glycine	5.17 ± 0.79	325

bilization conditions that extracted >25% of the protein from brain membranes were required in order to extract the PCP receptor from membranes. This necessitated the use of CHAPS concentrations in excess of 10 mM (relative to a final membrane protein concentration during solubilization of 10 mg/ml). Significant levels of specific [^{3}H]TCP or [^{3}H]MK-801 binding were restored to these samples only after their lipid content was enhanced by supplementation with vesicles prepared from a brain lipid extract and dilution of the CHAPS concentration well below its effective concentration.[3] The data shown in Fig. 3 further demonstrate that the recovery of solubilized receptor binding activity is highly dependent upon the concentration of brain lipid added to the membrane extracts.

The specific and saturable binding of [^{3}H]TCP and [^{3}H]MK-801 to vesicle preparations of the solubilized and reconstituted PCP receptor is shown in Fig. 4, A and B, respectively. Scatchard plots of these data (Fig. 4, A and B, *insets*) demonstrate that a single, high affinity binding site was recovered in each case. The K_d for [^{3}H]TCP binding was 15 ± 3 nM; the comparable value for [^{3}H]MK-801 binding in the vesicle preparations was 13.9 ± 3.1 nM. The calculated B_{max} values of 5.1 ± 2 pmol/mg and 8.5 ± 1.7 pmol/mg for this experiment represent 5–12-fold enrichments in the high affinity binding activities, respectively. The partial purification of activity observed is due to the fractionation of membrane protein that occurs during

[3] The central micellar concentration of CHAPS is between 6 and 10 mM in 0–0.5 M Na^+ at 20–25° (34).

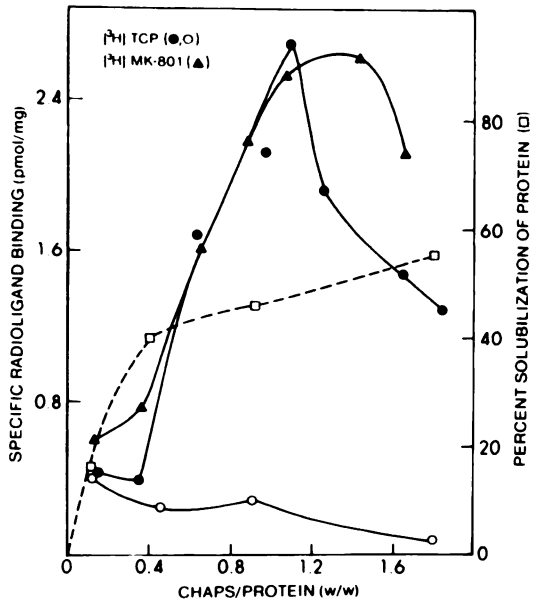

Fig. 2. Solubilization of the PCP receptor from rat brain membranes. Specific radioligand binding was determined using a [^{3}H]TCP concentration of 10 nM (●, ○) or [^{3}H]MK-801 concentration of 5 nM (▲), after dilution of detergent extracts in buffer B (○) or after reconstitution in brain lipid vesicles (●, ▲). The percentage of solubilization of protein (□) refers to the fraction of membrane protein recovered in supernatants after the ultracentrifugation of detergent extracts. Detergent extracts were obtained by incubating membranes with variable (0–30 mM) concentrations of CHAPS. The reconstitution and assay methodology used is described in Experimental Procedures. The data are representative of triplicate experiments.

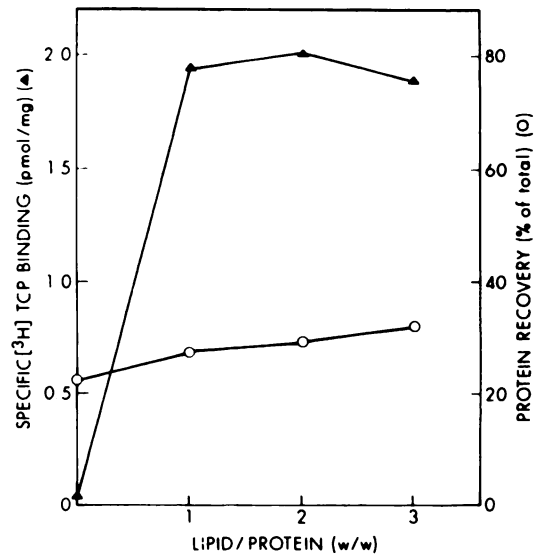

Fig. 3. Lipid-dependent reconstitution of PCP receptor binding. Varying amounts of brain lipid vesicles were added to fixed amounts of a detergent extract obtained by solubilization of membranes with 10 mM CHAPS. Specific binding to the vesicle preparations (▲) was determined using a [^{3}H]TCP concentration of 10 nM. The protein recovery (○) refers to the yield of protein in the sedimenting vesicles. The reconstitution and assay methodology used is described in Experimental Procedures. The data are representative of triplicate experiments.

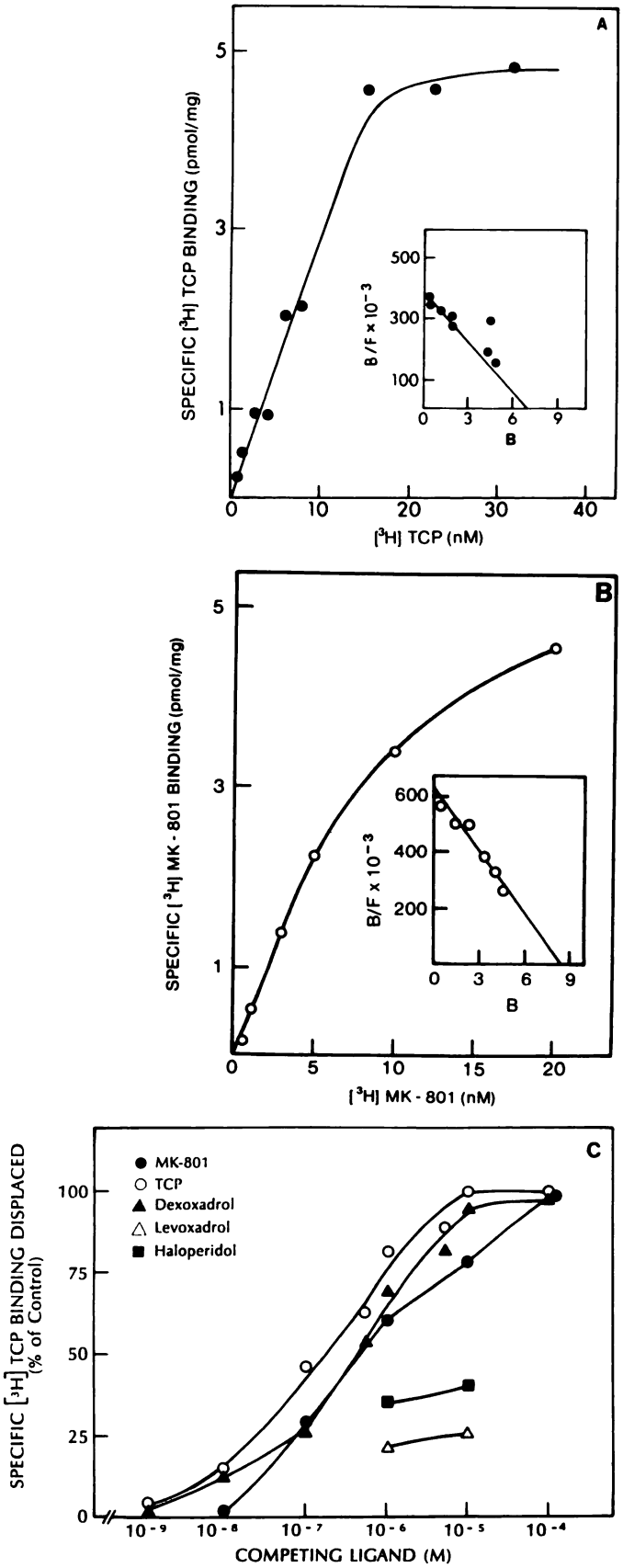

Fig. 4. Characterization of the reconstituted PCP receptor. Solubilized/reconstituted receptor preparations were obtained by solubilization of membranes with 10 mM CHAPS and reconstitution of extracts in brain lipid vesicles. A, Specific [^{3}H]TCP binding. *Inset*, Scatchard plot of these data. B, Specific [^{3}H]MK-801 binding. *Inset*, Scatchard plot of these data. C, The displacement of [^{3}H]TCP binding from vesicle preparations by MK-801 (●), TCP (○), dexoxadrol (▲), levoxadrol (△), and haloperidol (■) is presented as the fractional displacement of total specific binding measured for 10 nM [^{3}H]TCP. The reconstitution and assay methodology used is described in Experimental Procedures. The data are representative of triplicate experiments.

centrifugation steps following the detergent solubilization of membranes and reconstitution of solubilized protein into vesicles. Examples of these protein recoveries are shown in Figs. 2 and 3. The incomplete protein recovery following reconstitution (Fig. 3) likely reflects the initial solubilization from membranes of peripherally associated membrane proteins that were not incorporated into the vesicle preparations. The protein recovery improved as higher CHAPS concentrations were used initially to solubilize a greater proportion of the hydrophobic membrane protein (data not shown). The results obtained in these binding studies routinely reflected a 70–100% recovery of [^{3}H]TCP- and [^{3}H]MK-801-binding activity in the vesicle preparations of the original high affinity binding interaction present in brain membranes. The increasing yield of solubilized receptors with increasing detergent concentration was matched by an equivalent reduction in the membrane-associated activity (data not shown). In contrast, in the absence of a reconstitution strategy only a small fraction of this binding activity was recovered by dilution of extracts in the assay to a level that does not interfere with measurements of specific ligand binding; these data are shown in Fig. 2. Various detergents, including CHAPS, inhibit binding to PCP receptors (24, 25); in this study, the direct binding of radioligand to receptor in extracts was not successfully measured in the presence of the significant detergent concentrations that were required for receptor solubilization.

Several additional reconstitution criteria established by Scheideler and Zukin (30) were also demonstrated for the solubilized/reconstituted PCP receptor. 1) The high levels of radioligand binding achieved in these preparations were not due to the specific binding of [^{3}H]TCP or [^{3}H]MK-801 to lipid vesicles. Further, ligand binding increased linearly over a range (0–0.1 mg/ml) of sample protein concentrations. 2) Negative staining/electron microscopy confirmed that uniformly sized proteoliposomes, 100–200 nm in diameter, were formed when the solubilized receptor was reconstituted (data not shown). 3) The lipid dependency shown in Fig. 3 is not a simple requirement for a bilayer. Only 17% of the ligand-binding activity normally restored after the incorporation of solubilized PCP receptors into brain lipid vesicles was obtained after substitution of a negatively charged, vesicle-forming, brain phospholipid (cardiolipin) in the reconstitution. No specific ligand binding was measured when a positively charged brain phospholipid (phosphatidylcholine) was used. Thus, specific lipids present in the brain lipid preparation used to make vesicles are required for receptor activation.

The displacement of specific [^{3}H]TCP binding to the solubilized/reconstituted receptor by selective ligands is shown in Fig. 4C, and the results are summarized in Table 1. Ligand binding to the vesicle preparations was stereoselective, in that dexoxadrol exhibited a >40-fold greater affinity for the receptor than did levoxadrol. Dexoxadrol, TCP, and MK-801 each effectively competed with [^{3}H]TCP for binding, over a comparable range of concentrations. IC_{50} values for these ligands were shifted to higher values, relative to corresponding values measured in membranes. The loss in affinity was more pro-

nounced as the detergent concentration used to solubilize membranes was increased (data not shown). This suggests that CHAPS partially dissociates protein-protein interactions, during membrane solubilization, that are crucial to the integrity of the PCP binding site. However, IC_{50} values for the solubilized/reconstituted preparations were still low, in comparison with the concentrations (>1 μM) of the σ receptor ligand haloperidol required to significantly displace [^{3}H]TCP binding.

The addition of glutamate to the assay enhanced [^{3}H]TCP binding in a concentration-dependent fashion (Table 2). The addition of 1 μM glutamate to the assay produced a 151% enhancement in specific binding, whereas an increase of 230% was achieved in the presence of 100 μM glutamate. However, the level of stimulation produced only at high glutamate concentrations could be matched and exceeded by the addition of increasing concentrations of glycine together with 1 μM glutamate. The marked improvement in the stimulation of [^{3}H]TCP binding over that observed in membranes is likely a reflection of the comparative difficulty in removing endogenous amino acids from synaptosomal membrane preparations.

Taken together, these results demonstrate the recovery of a high affinity PCP receptor from brain. The synergistic action of glutamate and glycine in enhancing radioligand binding further suggests that an association of this site with the NMDA receptor complex has been maintained in the vesicle preparations.

Photoaffinity labeling of the solubilized/reconstituted PCP receptor. Vesicle preparations that possessed enhanced levels of the partially fractionated PCP receptor binding activity were used in experiments aimed at photolabeling receptor binding site polypeptides with azido-[^{3}H]PCP; this allowed us to eliminate the considerable low affinity and nonspecific labeling of polypeptides that occurs in membranes (5, 27, 28). The photoreactive, azido-[^{3}H]PCP analog labeled only two polypeptides (M_r 98,000 and 59,000) in the vesicle preparations (Fig. 5). Further, the covalent association of azido-[^{3}H]PCP with both proteins was substantially reduced by incubation of assay samples with an excess of PCP before photolysis by exposure to UV light (Fig. 5). The larger of the polypeptides labeled in this study corresponds in size to membrane proteins of M_r 90,000 (5, 27) and 98,000 (28) that were previously affinity labeled with azido-[^{3}H]PCP in brain membranes. The smaller, M_r 59,000, polypeptide has also been labeled in brain membranes (5, 27). It may be a second subunit required for receptor/ion channel reconstitution or a proteolytic fragment of the larger polypeptide that is labeled.

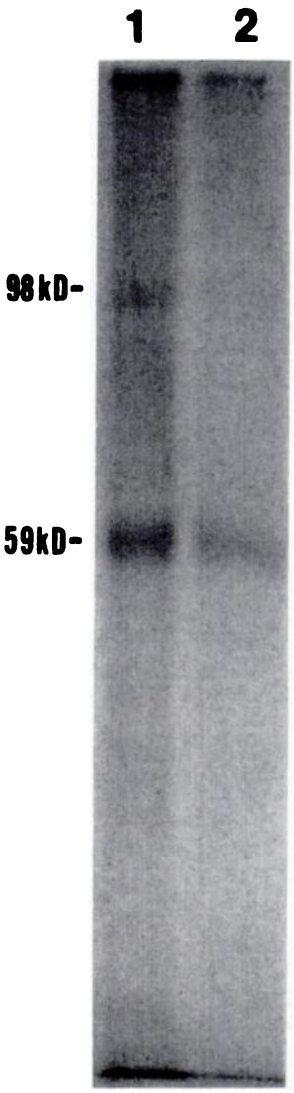

Fig. 5. Photoaffinity labeling of the reconstituted PCP receptor. Vesicle preparations containing high levels of reconstituted PCP receptor binding activity were incubated with 20 nM azido-[^{3}H]PCP for 1 hr in the dark, in the absence (*lane 1*) or presence (*lane 2*) of 100 μM PCP, and were irradiated with UV light. Labeled polypeptides were resolved by SDS-polyacrylamide gel electrophoresis and identified by autoradiography. The solubilization/reconstitution, cross-linking, and assay methodology used is described in Experimental Procedures. The data are representative of duplicate experiments.

Discussion

The recognition elements that govern ligand binding to the high affinity PCP binding sites present in brain are largely uncharacterized. Thus far, kinetic data from binding studies performed using membranes (18, 19) have been combined with electrophysiological data from whole-cell recordings (20), to infer a mechanism of PCP binding to the nonselective cation channel associated with the NMDA receptor. However, unequivocal identification of the receptor subunit(s) forming the channel-associated binding site has been especially difficult due to the mixture of high and low affinity PCP binding sites present in brain membranes and the comparatively low number of total sites. The inability of several laboratories, including our own, to restore ligand binding to PCP receptors after membrane solubilization further suggests that receptor reactivation is lipid dependent.

In this study, we established conditions that have allowed us to reactivate ligand binding by incorporating solubilized PCP receptors into vesicles prepared from a total brain lipid extract. A high affinity PCP binding site was successfully solubilized from membranes in a form that is intrinsically stable but inactive in CHAPS. In binding studies with radiolabeled TCP, we now show that specific ligand binding activity can be restored to these cell membrane extracts via detergent removal and lipid readdition. A high efficiency of reconstitution was observed; nearly quantitative levels of receptor binding activity were recovered in the vesicle preparations. Incorporation of the solubilized receptor into a bilayer formed from different, pure, vesicle-forming phospholipids was not sufficient to reconstitute ligand binding activity; this suggests that the lipid dependency

is met by minor lipid cofactors and/or a combination of major lipids present in the brain lipid preparation used in the reconstitution. A similar finding for the voltage-sensitive sodium channel has been reported by Agnew and Raftery (22). Identification of the activating species will, however, require an extensive systematic investigation of the specific lipid dependency.

Competition binding experiments were used to characterize the solubilized and reconstituted receptor pharmacologically. Several findings suggest that the [^{3}H]TCP binding site detected in lipid vesicles is the high affinity PCP receptor associated with the NMDA receptor/ion channel complex in brain membranes. 1) Drugs related to PCP displaced specific [^{3}H]TCP binding to the vesicle preparations, in a stereospecific manner. 2) Ligands selective for the NMDA receptor, i.e., TCP and the anticonvulsant MK-801, effectively displaced specific radioligand binding to the solubilized/reconstituted receptor over a similar concentration range. 3) The neuroleptic drug haloperidol was comparatively ineffective at displacing binding to the vesicle preparations. 4) Specific [^{3}H]TCP binding to the solubilized/reconstituted receptor was potentiated by glutamate and glycine, in a dose-dependent and additive manner.

Shifts in IC_{50} values measured for each ligand tested were noted in displacement studies, relative to values established in membranes. These changes are consistent with the binding of PCP receptor ligands within the ion channel and partial dissociation of the channel-forming subunits during detergent solubilization. Alternatively, the presence of residual CHAPS may interfere with ligand binding to the reconstituted receptor.

Photoaffinity labeling experiments provided evidence suggesting that the high affinity PCP binding site is formed by the association of heterologous receptor subunits. The solubilized/reconstituted samples used in these studies were prepared using conditions that restored increased levels of specific ligand-binding activity to the partially fractionated receptor. Only two PCP binding site polypeptides (M_r 98,000 and 59,000) were affinity labeled with azido-[^{3}H]PCP in these vesicle preparations; this result contrasts sharply with previous work demonstrating cross-linking of this ligand to multiple membrane polypeptides (5, 27, 28). The labeling of each membrane protein in this study was mostly prevented by an excess of competing unlabeled drug, confirming that the photoreactive ligand selectively interacts with the reconstituted PCP receptor. Our ability to quantitatively reconstitute high affinity PCP receptors after membrane solubilization further argues that the M_r 98,000 and 59,000 affinity-labeled receptor polypeptides present in these partially fractionated preparations are sufficient to restore ligand-binding activity at this site. In contrast, Ikin *et al.* (35) did not label a M_r 98,000 polypeptide with azido-[^{3}H]PCP after the affinity sorting of solubilized PCP receptors. This result may reflect the incomplete recovery of receptor subunits, because only a small fraction of the original membrane-associated PCP binding activity was recovered in the absence of a reconstitution strategy. Using azido-[^{3}H]MK-801, Sonders *et al.* (36) have labeled a M_r 120,000 polypeptide in brain homogenates; this size agrees well with the target size of M_r 118,000 determined by radiation inactivation of the [^{3}H]TCP binding site (37). The difference in size between the M_r 120,000 and 98,000 polypeptides is not large and may be due to differences in sample preparation, which result in anomolous migration behavior on gels, or partial proteolysis of one or both of these polypeptides. Alternatively, azido-[^{3}H]MK-801 may bind to a different channel-forming subunit of approximately the same size that is not labeled by azido-[^{3}H]PCP.

It is noteworthy that membrane-associated subunits of two other ligand-gated ion channels are similar in size to the M_r 95,000 and 59,000 polypeptides labeled in this study. The two proteins encoded by the cloned kainate-binding glutamate receptor sequences (M_r 100,000 and 48,000) both bind kainate when expressed (36–38); the larger of the two polypeptides forms a functional ion channel. Affinity-purified integral membrane proteins of M_r 58,000 and 48,000 form the chloride channel associated with the strychnine-sensitive glycine receptor (39).

In summary, solubilization conditions were used that allowed us to efficiently extract a high affinity PCP receptor from brain membranes with the zwitterionic, bile salt, detergent CHAPS. A reconstitution approach was then used to restore high levels of radioligand-binding activity to samples that otherwise possessed little intrinsic activity. These partially fractionated vesicle preparations have proven to be highly suitable for use in studies aimed at investigating the identity of membrane-associated lipid and protein determinants required for PCP receptor function. The goal of future studies in the laboratory will be to test whether ion channel activity and drug binding sites for NMDA receptor ligands can be localized to the M_r 98,000 and 59,000 PCP-binding polypeptides.

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