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N-Methyl-D-aspartate/Phencyclidine Receptor Complex of Rat Forebrain: Purification and Biochemical Characterization†

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ABSTRACT: The *N*-methyl-D-aspartate (NMDA)/phencyclidine (PCP) receptor from rat forebrain was solubilized with sodium cholate and purified by affinity chromatography on amino-PCP–agarose. A 3700-fold purification was achieved. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and dithiothreitol revealed four major bands of M_r 67 000, 57 000, 46 000, and 33 000. [^{3}H]Azido-PCP was irreversibly incorporated into each of these bands after UV irradiation. The dissociation constant (K_d) of [1-(2-thienyl)cyclohexyl]piperidine ([^{3}H]TCP) binding to the purified NMDA/PCP receptor was 120 nM. The maximum specific binding (B_{max}) for [^{3}H]TCP binding was 3.3 nmol/mg of protein. The pharmacological profile of the purified receptor complex was similar to that of the membranal and soluble receptors. The binding of [^{3}H]TCP to the purified receptor was modulated by the NMDA receptor ligands glutamate, glycine, and NMDA.

The *N*-methyl-D-aspartate (NMDA)[1] type of excitatory amino acid receptor is one of the best characterized glutamate receptors in the mammalian central nervous system. These receptors, which are ligand-gated cation channels, are involved in synaptic plasticity (Artola & Singer, 1987; Collingridge, 1987) and in long-lasting enhancement of synaptic efficacy (i.e., long-term potentiation), thought to be the basis of processes involved in learning and memory (Collingridge et al., 1983; Collingridge & Bliss, 1987). Overactivation of these receptors is associated with epileptogenic seizures (Turski et al., 1985), neurotoxicity (Rottman & Olney, 1987), and neuronal loss due to hypoglycemia (Rottman & Olney, 1987) and ischemia (Cotman & Iversen, 1987; Kemp et al., 1987).

The role of dissociative anesthetics, such as PCP and related drugs, as open-channel blockers of NMDA receptors (Honey et al., 1985; Foster & Wong, 1987; Kloog et al., 1988), as well as the notion of a high-affinity NMDA/PCP receptor complex, is well established. Therefore, in recent years PCP and its analogues have been used as biochemical probes of NMDA receptor channel activation.

We recently described the successful solubilization of high-affinity PCP-binding sites from rat forebrain membranes, utilizing the anionic detergent sodium cholate (Ambar et al., 1988). The binding of [^{3}H]TCP, a potent PCP analogue, was shown to be modulated by NMDA receptor ligands, thus providing further evidence for the existence of an NMDA/PCP receptor complex. We now describe the purification of the solubilized NMDA/PCP receptor complex by affinity chromatography.

Experimental Procedures

Materials. [^{3}H]TCP (28.6 Ci/mmol) and [^{3}H]AZ-PCP (16.8 Ci/mmol) were purchased from Israel Nuclear Center (Negev, Israel). Dexoxadrol and levoxadrol were donated by Dr. A. E. Jacobsen (National Institutes of Health). MK-801 was a gift from Merck Sharp & Dohme Research Laboratories. PCP and NH_2-PCP were a gift from Dr. A. Kalir (Tel-Aviv University). The synthesis of NH_2-PCP has been described by Kalir et al. (1978). NMDA and DL- and D-AP-5

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[1] Abbreviations: NMDA, *N*-methyl-D-aspartate, PCP, phencyclidine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*NN*′,*N*′-tetraacetic acid; AP-5, D-(−)-2-amino-5-phosphovalerate; AZ-PCP, *N*-[1-(3-azidophenyl)cyclohexyl]piperidine; PMSF, phenylmethanesulfonyl fluoride; TCP, [1-(2-thienyl)cyclohexyl]piperidine; Glu, glutamate; Gly, glycine; EC_{50}, concentration causing 50% of maximal effect; DTT, dithiothreitol.

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were purchased from Cambridge Research Biochemicals. Sodium cholate was obtained from Serva and Sigma. (*p*-Nitrophenyl)agarose (containing 25–40 μmol of PNP/mL of gel) was obtained from Sigma. All other materials were from Sigma.

Preparation of Amino-PCP–Agarose. (*p*-Nitrophenyl)-agarose (40 mL) in 2-propanol was well washed with 100% 2-propanol on a sinter funnel. An aqueous solution of ε-aminocaproic acid, which forms the spacer arm, was added at a final concentration of 0.25 M in pH 7.6–8.0. The mixture was shaken for 72 h at 4 °C. The gel was then washed with water and 0.2 N NaOH to remove all traces of *p*-nitrophenol and again with water and 0.3 N HCl. Acid was removed with water and the gel was then washed with increasing concentrations of dioxan (30%, 60%, 100%). The matrix was suspended in a solution of *p*-nitrophenol in dioxane at a concentration of 1 mmol/1 mL of gel in the presence of the same concentration of dicyclohexylcarbodiimide. The mixture was shaken overnight at room temperature. The matrix was then washed with dioxane and 2-propanol, and amino-PCP was added at a 5- to 10-fold excess over the concentration of active ester on the matrix. The mixture was shaken at room temperature until the coupling of amino-PCP was completed (~10–12 days). The gel was then washed with water and 20 mM Tris-HCl, 2 mM EDTA, and 0.05% sodium cholate, pH 7.4, and packed into a glass column. The concentration of ligand bound to the matrix was estimated according to the concentration of *p*-nitrophenolate released in the coupling reaction and was found to be about 8 μmol/mL of gel.

Preparation of Solubilized Receptor. Forebrains were removed from Charles Rivers derived male rats and homogenized in 20 volumes of ice-cold 50 mM Tris-HCl buffer, pH 7.4, containing 0.32 M sucrose, 1 mM EGTA, 3 mM EDTA, and a mixture of protease inhibitors, 0.1 mM PMSF, 5 units/mL aprotinin, and 5 μg/mL pepstatin A, in a glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 1000*g* for 10 min, and the supernatant was then centrifuged for 20 min at 20000*g*. The pellet was resuspended with a glass–Teflon homogenizer in 20 mM Tris-HCl, pH 7.4, and 2 mM EDTA containing the above mixture of protease inhibitors, to a final concentration of 8 mg of protein/mL. Protein concentration was determined according to the method of Lowry et al. (1951). This membrane suspension was mixed with an equal volume of 3% sodium cholate in 5 mM Tris-HCl, pH 7.4, to a final detergent concentration of 1.5% and 4 mg of protein/mL. The mixture was shaken for 1 h at 4 °C and centrifuged at 100000*g* for another hour. The supernatant was then dialyzed for 4 h against 500 volumes of 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, and 0.1 mM PMSF, to remove the detergent which inhibits binding of PCP-like ligands. The resulting dialysate was used as the solubilized receptor preparation.

Affinity Chromatography on Amino-PCP–Agarose. The affinity column was preequilibrated with 20 mM Tris-HCl, pH 7.4, 0.05% sodium cholate, 2 mM EDTA, and the above mixture of protease inhibitors, (buffer A). The soluble extract was applied to the affinity column (5.5 cm × 1.5 cm) at 60 mL/h by using a peristaltic pump. After being loaded, the column was washed with 40 mL of buffer A containing a 10^{-4} M concentration of the competitive glutamate antagonist DL-AP-5. The column was then washed overnight with 800 mL of buffer A containing 10^{-5} M DL-AP-5. The receptor was eluted with 36–40 mL of buffer A containing 10 μM Glu, 1 μM Gly, and 10 μM PCP. The eluate was subjected to dialysis for 4 h against 200–500 volumes of buffer A (containing only 0.1 mM PMSF as a protease inhibitor) to remove free PCP. The dialysis buffer was exchanged every hour. The whole purification procedure was carried out at 4 °C.

In order to facilitate purification of large amounts of preparation, and to increase receptor yield, two similar columns were used simultaneously and their eluates combined.

After each elution the columns were regenerated by using solutions of 0.5 M NaCl with 0.1 M CH_3COOK and 0.5 M NaCl with 0.1 M $KHCO_3$. After being used several times, the matrix was washed with 6 M guanidinium chloride.

Receptor-Binding Assay. Membranal and soluble preparations were assayed as described in detail previously (Ambar et al., 1988), by use of the rapid filtration assay. The same assay was used for purified receptor preparations. Purified receptors (200 μL) were incubated with 50 μL of [^{3}H]TCP in the absence (total binding) or presence (nonspecific binding) of 50 μL of PCP at a final concentration of 10^{-4} M. The final reaction volume was 300 μL. All ligands were prepared in 5 mM Tris-HCl (pH 7.4). Final [^{3}H]TCP concentration was 30 nM. Equilibrium binding assays were conducted in the presence of 1 μM glutamate and 1 μM glycine, and for an incubation time of 1 h at 25 °C. The effect of NMDA receptor ligands on [^{3}H]TCP binding was studied by using an incubation time of 10 min at 25 °C. Binding reactions were terminated by the addition of 3 mL of ice-cold 5 mM Tris-HCl buffer (pH 7.4), followed by rapid filtration over GF/F filters (Tamar, Jerusalem, Israel) pretreated with 0.05% poly(ethylenimine). The filters were washed three more times with 3 mL of the buffer and counted for radioactivity in 4 mL of scintillation liquid (Hydro Luma, Lumac Systems, Netherlands). Purified receptor assays were performed in six replicates.

Protein concentrations in membranal and soluble preparations were determined by the method of Lowry et al. (1951). In the purified preparation protein was determined by using the sensitive silver-binding assay (Krystal, 1987), which permits detection of protein in the nanomolar range. It was necessary to concentrate the purified preparation 10-fold prior to protein determination.

SDS–Polyacrylamide Gel Electrophoresis. Purified receptor preparations were concentrated under nitrogen by using Omegacell filters (Bio Lab Laboratories) with a membrane cutoff of 10K and Centricon 10 filters (Amicon) with a 10K cutoff. Samples were then dissolved in sample buffer containing 2% SDS, 10% glycine, 10 mM DTT, 52.5 mM Tris-HCl, pH 6.8, and bromophenol blue at a sample to buffer ratio of 2:1. Samples were boiled for 5 min. SDS–PAGE was performed with slab gels according to Laemmli (1970), using a 10% (w/v) polyacrylamide gel.

The receptor polypeptide pattern was viewed after silver staining.

Photoaffinity Labeling. Photoaffinity labeling of the purified receptor preparation with [^{3}H]AZ-PCP was performed essentially as described by Haring et al. (1986, 1987). Aliquots (200 μL) of purified preparation were incubated with 50 μL of [^{3}H]AZ-PCP at a final concentration of 100 nM in 50 μL of 5 mM Tris-HCl buffer, pH 7.4, containing 10^{-5} M glutamate (total binding) or with 50 μL of PCP at a final concentration of 10^{-4} M in the same buffer (nonspecific binding). Following an incubation period of 1 h or 5 min at 25 °C in the dark, the reaction mixture was photolyzed with a long-wave ultraviolet spotlight lamp (Thomas Scientific Apparatus, Model B-100A, 366 nm) at a distance of 5 cm (1500 μW/cm^2) with continuous stirring for 5 min. Assays were performed in triplicate, after which the triplicate samples were combined,

Table I: Purification of the NMDA/PCP Receptor Complex[a]

fraction	vol (mL)	[^{3}H]TCP-binding sites (pmol)[b]	[^{3}H]TCP binding sites, cor (pmol)[c]	protein (mg)	specific activity (pmol/mg)	degree of purification	yield (%)
crude membrane	42	265	530	336.5	1.57	1.0	100.0
sodium cholate extract	84	66	132	218.2	0.60	0.38	24.0
affinity-purified receptor	36	7.6	38	0.0065	5846	3732.6	7.0

[a] Purification was carried out as described under Experimental Procedures. [b] Binding to all preparations was determined by the filtration assay over GF/F filters at 30 nM [^{3}H]TCP as described. [c] [^{3}H]TCP-binding sites were corrected for the apparent K_d values according to the equation: $B/B_{max} = [L]/(K_{d,app} + [L])$. The apparent K_d values were derived from Scatchard plots. In the case of the purified receptor no correction was introduced for the presence of PCP (see Tabel II). The K_d values for the three preparations were 30 nM (crude), 30 nM (soluble), and 120 nM (purified). These values were used to calculate specific activities, degrees of purification, and yields.

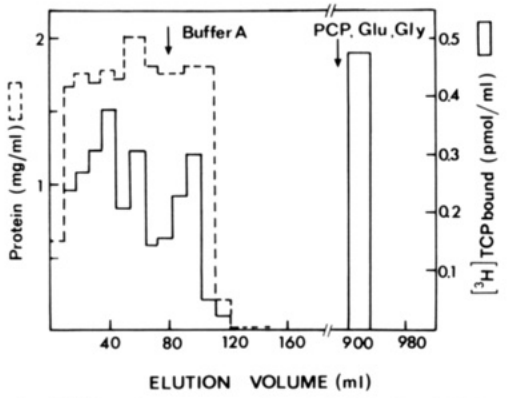

FIGURE 1: Affinity chromatography of the NMDA/PCP receptor complex on amino-PCP–agarose. Solubilized NMDA/PCP receptor (66 pmol in 83 mL), prepared as described under Experimental Procedures, was applied to a column containing 9 mL of amino-PCP–agarose at a flow rate of 60 mL/h. The column was then washed with 800 mL of buffer A containing 10^{-5} M DL-AP-5 and eluted with buffer A containing 10 μM glutamate, 1 μM glycine, and 10 μM PCP as described under Experimental Procedures. The binding of [^{3}H]TCP to samples containing PCP was determined after dialysis against buffer A as described under Experimental Procedures. The recovery of [^{3}H]TCP-binding sites in the PCP eluate was 11.5%.

concentrated to ca. 80 μL on a Centricon 10 filter, and prepared for SDS–PAGE as described above.

After electrophoresis, protein was fixed on the gel by Coomassie blue staining. Gels were sliced into 2-mm slices with a gel slicer. Each slice was digested in 5 mL of Lipoluma–Lumasolve–water (10:1:0.2) (Lumac) in a closed scintillation vial. Radioactivity was determined after 24 h by scintillation spectrometry.

Results

Purification of the NMDA/PCP Receptor Complex. The NMDA/PCP receptor complex was purified from a sodium cholate extract of crude membranes from rat forebrain by affinity chromatography on amino-PCP–agarose.

As shown in Figure 1, between 50 and 60% of the [^{3}H]-TCP-binding sites were not retained by the affinity column and were recovered in the flow-through together with the main bulk of protein. No additional [^{3}H]TCP sites were detected after further washings of the column with 800 mL of buffer A containing 10^{-5} M DL-AP-5. About 28% of the [^{3}H]TCP-binding sites were specifically recovered upon addition of 10 μM PCP, 10 μM glutamate, and 1 μM glycine. This value is low relative to the amount of ligand coupled to the column ($\sim$8 μmol/mL) and is mainly due to the rapid dissociation of receptors from the affinity matrix.

The receptor was assayed after the PCP used for elution had been removed from the solution by dialysis.

Data from a typical purification experiment are summarized in Table I. The recovery of [^{3}H]TCP-binding sites after solubilization was 24%, with a 60% decrease in specific activity.

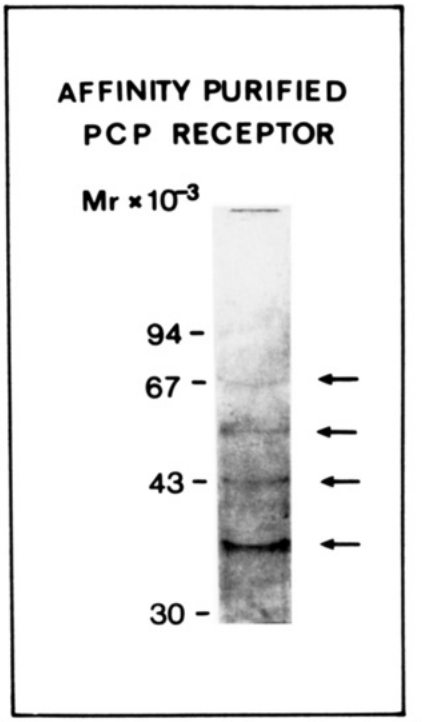

FIGURE 2: SDS–polyacrylamide gel electrophoresis of the purified NMDA/PCP receptor complex. [^{3}H]TCP-binding sites were solubilized and purified as described under Experimental Procedures. Purified preparation (20 mL, $\sim$5 pmol of [^{3}H]TCP-binding sites) was concentrated as described under Experimental Procedures. The sample was electrophoresed, and the gel was silver stained. Molecular weights were determined by using molecular weight markers.

The yield of purified receptor obtained from 10 rat forebrains (530 pmol of binding sites, 336 mg of protein) was 7.6 pmol of [^{3}H]TCP-binding sites, as determined by filtration assay with 30 nM [^{3}H]TCP (38 pmol after correction for the apparent K_d of [^{3}H]TCP). Purification was 3723-fold, with a total purified receptor yield of 7% and a purity of 5846 pmol/mg of protein. Receptor purity in different experiments ranged between 2500 and 6000 pmol/mg of protein.

SDS–PAGE of the purified NMDA/PCP receptor complex after silver staining revealed four major bands of M_r 67K, 57K, 46K, and 33K (Figure 2).

Three polypeptides of M_r 67–68K, 52–57K, and 42K were specifically and irreversibly labeled by the affinity probe [^{3}H]AZ-PCP (Figure 3). The 52K and 42K polypeptides were more strongly labeled than the 67K polypeptide. It was occasionally possible to obtain a labeled peak at M_r 33–35K in samples that had been subjected to the short incubation time of 5 min.

It may therefore be concluded that these polypeptides are associated with the NMDA/PCP receptor channel.

Binding Properties of the NMDA/PCP Receptor Complex. The binding properties of the purified preparation were de-

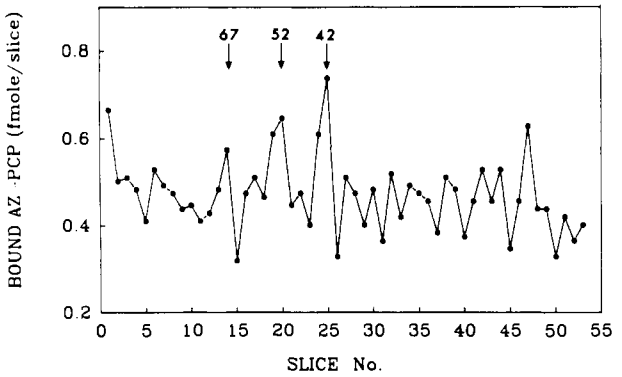

FIGURE 3: Photoaffinity labeling of the purified NMDA/PCP receptor complex. Purified NMDA/PCP receptor was photoaffinity-labeled with [^{3}H]AZ-PCP and then subjected to SDS–polyacrylamide gel electrophoresis as described under Experimental Procedures. After Coomassie blue staining, the gel was cut into 2-mm slices, digested in a mixture of Lipoluma–Lumasolve, and counted for radioactivity by scintillation spectrometry. Arrows indicate molecular weights ($\times 10^{-3}$) of the different labeled polypeptides.

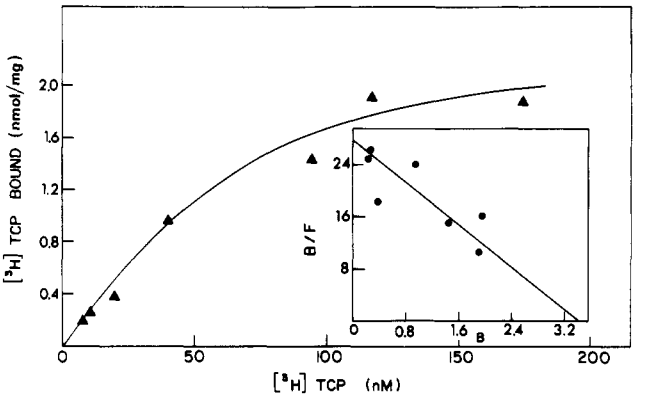

FIGURE 4: Equilibrium binding of [^{3}H]TCP to the isolated NMDA/PCP receptor complex. [^{3}H]TCP binding activity was measured by the filtration assay as described under Experimental Procedures, at [^{3}H]TCP concentrations between 5 and 250 nM. Inset: Scatchard analysis. Bound ligand (B) is expressed as nmol/mg of protein. Bound over free (B/F) is expressed as pmol/(mg of protein·nM).

termined by using the rapid filtration technique used in previous studies to assay the soluble receptor. Nonspecific binding of [^{3}H]TCP in the purified receptor assay did not exceed that observed for soluble or for membranal receptors under the same assay conditions. However, since receptor solutions after purification were very dilute on account of receptor loss at each purification step, there was a decrease in the signal-to-noise ratio (for example, at a concentration of 30 nM [^{3}H]TCP the nonspecific values for binding to membranal, soluble, and purified preparations were 30%, 50, and 70%, respectively). Efforts to lower this nonspecific adsorption were not successful. Attempts to concentrate the dilute receptor preparation, although the protein concentrations obtained were reasonable, resulted in a loss of binding activity. Thus we were unable to measure the association kinetics of [^{3}H]TCP binding to the purified receptors because of the low sensitivity of the assay at short incubation periods. Performing purified receptor assays in six replicates enabled a more accurate determination of the different binding parameters.

Using this procedure, it was possible to detect significant effects of glutamate and glycine on the binding of [^{3}H]TCP to the purified receptors by using incubation times of 5 and 10 min. A 2.5- to 4.6-fold increase of [^{3}H]TCP binding over basal was observed in the presence of 1 μM glutamate and 1 μM glycine. Equilibrium of [^{3}H]TCP binding in the presence of glutamate + glycine was reached after incubation for 45 min, as judged from the lack of increase in binding at longer incubation periods. Therefore, in all equilibrium binding assays an incubation period of 1 h was used and 1 μM glutamate + 1 μM glycine were included.

Figure 4 depicts a typical experiment showing the equilibrium binding of [^{3}H]TCP to the purified receptor. The ligand displays saturable binding to a single class of binding sites, with a dissociation constant (K_d) of 120 nM and a B_{max} value of 3.3 nmol/mg of protein, as determined by Scatchard analysis (Figure 4 inset).

All binding assays were performed on the day of elution, since the receptors lost a considerable percentage of their binding activity after 1 day at 4 °C and were completely inactive after freezing.

Pharmacological Characterization of the Purified NMDA/PCP Receptor Complex. In order to pharmacologically characterize the purified receptor complex, two types of experiments were performed: the interaction of noncompetitive blockers with the channel was examined by using competition binding experiments, and the effect of NMDA receptor ligands was studied by examining their ability to enhance [^{3}H]TCP binding.

Table II: Pharmacological Profile of the Purified NMDA/PCP Receptor Complex

ligand	K_{app} (nM)[a]	ED_{50}[b]
[^{3}H]TCP	60	
PCP	220	
MK-801	83	
dexoxadrol	113	
levoxadrol	610	
glutamate		150
glutamate + Gly		90
NMDA		500
NMDA + Gly		200
glycine		50
glycine + Glu		30

[a] K_{app} of [^{3}H]TCP was derived from the Scatchard plot in Figure 3 and corrected for the presence of residual PCP in the purified preparation. K_{app} of unlabeled ligands was derived from competition curves and similarly corrected. The PCP concentration remaining in the purified preparation after dialysis was estimated by using [^{3}H]PCP. [b] ED_{50} values were derived from dose–response curves such as those shown in Figure 5.

As shown in Table II, the different PCP analogues tested exhibited pharmacological specificities similar to those observed for membranal and solubilized receptors (Ambar et al., 1988). As can be seen from the apparent K_I values of the two stereoisomers dexoxadrol and levoxadrol, the stereoselectivity at the PCP-binding site was maintained after receptor purification. However, it should be pointed out that levoxadrol appears to have a significantly higher affinity toward the purified receptor (K_{app} = 610 nM) as compared with the solubilized receptor (K_{app} = 32000 nM) (Ambar et al., 1988). We have no explanation of this discrepancy as yet.

Figure 5 demonstrates the dependence of [^{3}H]TCP binding on NMDA receptor ligands. Both NMDA and glycine increased [^{3}H]TCP binding in a dose-dependent manner. Maximal enhancement of [^{3}H]TCP binding was obtained at 0.5–1 μM glycine and 1 μM NMDA. Glutamate produced similar effects. One micromolar glutamate produced a 1.5-fold increase over the maximal glycine-induced [^{3}H]TCP binding (Figure 5A). Gly had a similar effect on the NMDA-induced binding (Figure 5B). Neither ligand affected the EC_{50} value of the other.

These effects are similar to those observed for the binding of noncompetitive blockers to membranal receptors (Loo et

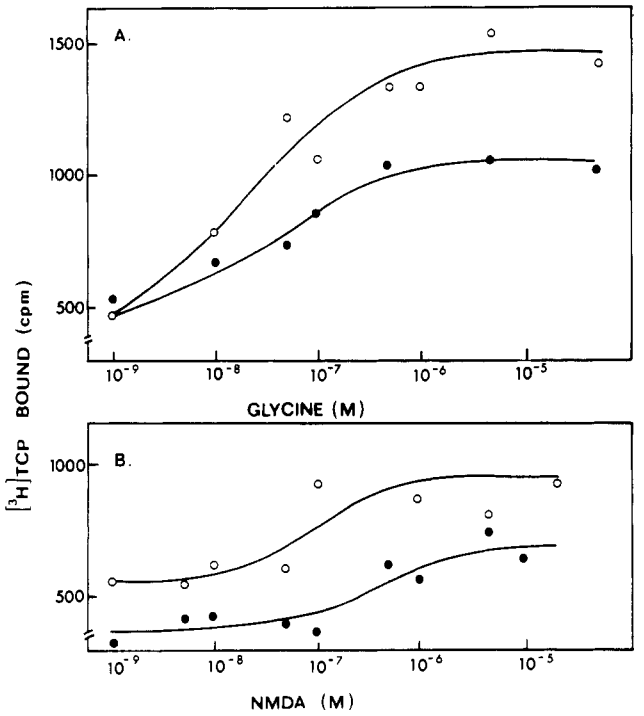

FIGURE 5: Effect of NMDA receptor ligands on the binding of [^{3}H]TCP to the purified NMDA/PCP receptor complex. Purified preparation (200 μL) was incubated with increasing concentrations of NMDA receptor ligands for 10 min, and the binding of [^{3}H]TCP was determined by the filtration assay as described under Experimental Procedures. (A) Dose–response to glycine in the absence (●) and presence (○) of 1 μM glutamate. (B) Dose–response to NMDA in the absence (●) and presence (○) of 1 μM glycine.

al., 1986; Foster & Wong, 1987; Johnson et al., 1987; Kloog et al., 1988).

Discussion

We describe here an affinity chromatographic procedure for purification of the NMDA/PCP receptor complex to apparent homogeneity within 12–15 h. The relatively short purification time, as well as the use of protease inhibitors throughout the isolation process, minimized proteolytic degradation and receptor inactivation. By means of this single purification step we were able to achieve purification of >3000-fold. The high degree of purification may be attributable to high-affinity binding of the retained receptors to the amino-PCP–agarose. This assumption is supported by the fact that receptors were retained by the column even after extensive washings. The low initial retention of receptors by the affinity matrix was probably due to the presence in the soluble extract of large amounts of endogenous glycine and glutamate which were not removed by dialysis; this would facilitate binding of the receptor to the amino-PCP matrix due to channel opening, but would also increase the dissociation of receptors from the matrix. Loading of the soluble preparation in the presence of the competitive glutamate antagonist AP-5 did not improve the yields, presumably because a larger fraction of the channels was now closed. Nevertheless, inclusion of AP-5 in the washing buffer secured the binding of the remaining receptors to the column; they were then eluted in the presence of glutamate and glycine.

The binding and pharmacological features of the purified receptor are similar to those observed in membranes and in soluble extracts. Purification did not significantly alter the binding affinities of the noncompetitive channel blockers, and the stereoselectivity of their binding site was unimpaired. The binding of [^{3}H]TCP to the purified complex exhibited a sensitivity to NMDA receptor ligands similar to that seen in membranes. The effect of glycine on glutamate-induced or NMDA-induced [^{3}H]TCP binding and the effect of glutamate on glycine-induced binding indicate that the entire NMDA/PCP receptor complex was functionally purified, since the interaction between the different ligand sites (glutamate, glycine) and the channel was maintained.

SDS–PAGE of the purified receptor followed by silver staining revealed a pattern of four polypeptides of M_r = 67 000 (α), 57 000 (β), 46 000 (γ), and 33 000 (δ). The labeled NMDA channel blocker [^{3}H]AZ-PCP irreversibly labeled three of these polypeptides, α, β, and γ, upon UV illumination. The largest amount of radioactivity was incorporated into the β (52K) and γ (42K) polypeptides. In some cases it was possible to detect a labeled peak corresponding to the 33 000 (δ) polypeptide usually when using short incubation periods (5 min). This result is consistent with the previously reported photoaffinity labeling of polypeptides by [^{3}H]AZ-PCP in crude membrane preparations (Haring et al., 1986, 1987), except for a 90K polypeptide labeled in the membranes but completely lacking in the purified preparation. Since [^{3}H]AZ-PCP, like all PCP-like ligands, binds to a specific site within the NMDA receptor channel, we may safely assume that the channel is formed by the four polypeptides labeled by this ligand and viewed by silver staining. The 33 000 polypeptide may be weakly associated with the channel, and its affinity labeling may depend on receptor conformation.

The molecular weight values of the four polypeptides add up to 203 000, which closely corresponds to the M_r of 209 000 ± 19 000 estimated for the NMDA receptor complex by the radiation inactivation method (Honore et al., 1987).

A polypeptide pattern such as that observed here is to be expected of a receptor as complex as the NMDA/PCP receptor. It should contain a binding site for glutamate/NMDA, a site for glycine which would interact allosterically with the Glu site, and, of course, the cation channel. All ligand-gated receptor channel complexes isolated up to now have proved to be oligomeric proteins with M_r values of 100 000–300 000 (Tallman & Gallager, 1985; Grenningloh et al., 1987; Guy & Hucho, 1987). It is therefore not surprising that the NMDA/PCP receptor complex should be a four-polypeptide oligomer. The similarity between the NMDA receptor channel and the nicotinic acetylcholine receptor channel, both being ligand-gated cation channels and blocked by PCP-like drugs (Oswald & Changeux, 1981; Haring et al., 1984; Foster & Wong, 1987), lends support to the suggestion that the four polypeptides detected here form the channel structure.

Further investigation is needed before the stoichiometric subunit composition of the NMDA/PCP receptor complex is established. In future studies the purified receptor should be incorporated into phospholipid vesicles in order to demonstrate its function as a cation channel.

The present work should facilitate the partial amino acid sequencing of the different polypeptides and the subsequent isolation and cloning of the relevant genes.

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Expression of the Human Multidrug Transporter in Insect Cells by a Recombinant Baculovirus

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ABSTRACT: The plasma membrane associated human multidrug resistance (*MDR*1) gene product, known as the 170-kDa P-glycoprotein or the multidrug transporter, acts as an ATP-dependent efflux pump for various cytotoxic agents. We expressed recombinant human multidrug transporter in a baculovirus expression system to obtain large quantities and further investigate its structure and mechanism of action. *MDR*1 cDNA was inserted into the genome of the *Autographa californica* nuclear polyhedrosis virus under the control of the polyhedrin promoter. *Spodoptera frugiperda* insect cells synthesized high levels of recombinant multidrug transporter 2–3 days after infection. The transporter was localized by immunocytochemical methods on the external surface of the plasma membranes, in the Golgi apparatus, and within the nuclear envelope. The human multidrug transporter expressed in insect cells is not susceptible to endoglycosidase F treatment and has a lower apparent molecular weight of 140 000, corresponding to the nonglycosylated precursor of its authentic counterpart expressed in multidrug-resistant cells. Labeling experiments showed that the recombinant multidrug transporter is phosphorylated and can be photoaffinity labeled by [^{3}H]-azidopine, presumably at the same two sites as the native protein. Various drugs and reversing agents (e.g., daunomycin > verapamil > vinblastine ≈ vincristine) compete with the [^{3}H]azidopine binding reaction when added in excess, indicating that the recombinant human multidrug transporter expressed in insect cells is functionally similar to its authentic counterpart.

The development of simultaneous resistance to multiple drugs represents a major obstacle to successful cancer chemotherapy and is often associated with enhanced expression of the human multidrug resistance (*MDR*1) gene [reviewed in Gottesman and Pastan (1988)]. The *MDR*1 gene encodes a 170-kDa protein, termed P-glycoprotein or the multidrug transporter, which is found abundantly on the external surface of multidrug-resistant cells (Willingham et al., 1987). The transfer of a cloned *mdr* cDNA into drug-sensitive cells is sufficient to confer the complete multidrug resistance phenotype (Gros et al., 1986a; Guild et al., 1988; Pastan et al., 1988; Ueda et al., 1987). On the basis of the *MDR*1 cDNA sequence, a structural model has been proposed which predicts a 1280 amino acid polypeptide chain containing 12 transmembrane regions, 2 nucleotide binding sites, and 3 potential N-linked glycosylation sites (Chen et al., 1986; Gros et al., 1986b).

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