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Identification of Polypeptides of the Phencyclidine Receptor of Rat Hippocampus by Photoaffinity Labeling with [^{3}H]Azidophencyclidine

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ABSTRACT: Polypeptide components of the phencyclidine (PCP) receptor present in rat hippocampus were identified with the photolabile derivative of phencyclidine [^{3}H]azidophencyclidine ([^{3}H]AZ-PCP). The labeled affinity probe was shown to reversibly bind to specific sites in the dark. The number of receptor sites bound is equal to those labeled by [^{3}H]PCP, and their pharmacology and stereospecificity are identical with those of the PCP/σ-opiate receptors. The dissociation constant of [^{3}H]AZ-PCP from these receptors is 0.25 ± 0.08 μM. Photolysis of hippocampus membranes preequilibrated with [^{3}H]AZ-PCP, followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, revealed the existence of five major labeled bands of which a M_r 90 000 band and a M_r 33 000 band were heavily labeled. Inhibition experiments, in which membranes were incubated with [^{3}H]AZ-PCP in the presence of various PCP analogues and opiates, indicate that labeling of both the M_r 90 000 band and the M_r 33 000 band is sensitive to relatively low concentrations (10 μM) of potent PCP/σ receptor ligands, while similar concentrations of levoxadrol, naloxone, morphine, D-Ala-D-Leu-enkephalin, atropine, propranolol, and serotonin were all ineffective. Stereoselective inhibition of labeling of the M_r 90 000 band and of the M_r 33 000 band was also observed by the use of dexoxadrol and levoxadrol. The M_r 33 000 band was not as sensitive as the M_r 90 000 band to inhibition by the selective PCP receptor ligands *N*-[1-(2-thienyl)cyclohexyl]piperidine and PCP. Strong inhibition of labeling of the M_r 33 000 band by less selective PCP receptor ligands such as *N*-[1-(3-hydroxyphenyl)cyclohexyl]piperidine and (±)-*N*-allylnormetazocine was also observed. The labeling of the other three polypeptides (M_r 62 000, 49 000, and 40 000) was only mildly affected by dexoxadrol and (±)-*N*-allylnormetazocine, suggesting that this interaction does not have a classical PCP/σ receptor pharmacology. Thus, these labeled bands could be constituents of a second PCP receptor.

Phencyclidine (PCP,[1] angel dust) is a synthetic drug introduced originally in the late 1950s as a general anesthetic (Johnston et al., 1959; Domino, 1964). Because of its prolonged duration of action and its psychotomimetic effects, it

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was withdrawn from clinical use, but it became a major drug of abuse (Raimey & Crowder, 1974). The above-mentioned effects as well as the schizophrenia-like psychotic syndrome it induced (Luby et al., 1962) prompted intensive investigation on the molecular mechanism of PCP action. The drug was shown to influence various biochemical processes as diverse as monoamine uptake (Johnson, 1978) and dopamine release (Johnson & Vickroy, 1981) and to interact with muscarinic (Kloog et al., 1977; Vincent et al., 1978; Gabrielevitz et al., 1980), nicotinic (Kloog et al., 1977; Kloog et al., 1979, 1980; Aronstam et al., 1980; Oswald & Changeux, 1981), and opioid receptors (Vincent et al., 1978; Su et al., 1980; Itzhak et al., 1981), butyryl- and acetylcholinesterases (Kloog et al., 1977; Maayani et al., 1974), and potassium channels (Blaustein & Itzkovicz, 1983; Albuquerque et al., 1981). It is not known which, if any, of these effects are related to the behavioral manifestations of PCP.

Zukin & Zukin (1979) and Vincent et al. (1979) have demonstrated the existence in brain homogenates of a specific binding site for [^{3}H]PCP whose displacement in vitro by PCP analogues correlates precisely with their relative potencies in behavioral tests. Quirion et al. (1981a), using slide-mounted sections of fresh-frozen rat brain and autoradiography, have presented similar results. Further studies on the [^{3}H]PCP binding site and its pharmacology and stereoselectivity (Hampton et al., 1982; Murray et al., 1982; Quirion et al., 1981b; Zukin et al., 1983; Mendelsohn et al., 1984) lent additional support to the idea that this receptor site is a highly relevant component in the manifestation of the drug's psychotomimetic effects. The interesting finding of features common to the psychotomimetic effects of PCP and benzomorphan opiates such as SKF 10047 and cyclazocine, as well as the displacement of [^{3}H]PCP binding by low concentrations of the latter drugs, led to the suggestion that PCP and the σ-opiates share a common site of binding and action (Quirion et al., 1981a; Hampton et al., 1982; Murray et al., 1982; Mendelsohn et al., 1984; Teal & Holtzman, 1980; Zukin et al., 1984). Although both the behavioral and the biochemical binding studies provide convincing evidence for a common PCP/σ receptor, we do not yet know which polypeptide(s) bear(s) the putative binding site of those ligands. Identification of the polypeptides bearing the PCP and the σ receptor binding sites should provide valuable information about the PCP/σ receptors. In this paper we describe the binding characteristics and affinity labeling of rat hippocampal PCP receptors, determined by employing the photoactivatable analogue of PCP *N*-[1-(3-azidophenyl)cyclohexyl]piperidine (azidophencyclidine, AZ-PCP). This drug was used in our previous studies to localize the site of noncompetitive blockers of cholinergic receptors from *Torpedo* electric organ (Haring et al., 1983a, 1984).

Materials and Methods

Materials

PCP and AZ-PCP were prepared as described previously (Haring et al., 1983a,b, 1984). [^{3}H]AZ-PCP (22.8 Ci/mmol) and [^{3}H]PCP (22.5 Ci/mmol) were purchased from Israel Nuclear Center, Negev, Israel. The purity of the drugs was >98%. [^{3}H]Morphine (45.1 Ci/mmol) and [^{3}H]ethylketocyclazocine (19.9 Ci/mmol) were purchased from New England Nuclear, Boston, MA. *N*-[1-(3-Hydroxyphenyl)cyclohexyl]piperidine (hydroxy-PCP) and *N*-[1-(2-thienyl)cyclohexyl]piperidine (TCP) were prepared as described previously (Haring et al., 1983b). (+)-Ketamine and (−)-ketamine were a gift from Dr. A. Kalir, Tel Aviv University. Dexoxadrol, levoxadrol, and (±)-SKF 10047 were donated by Dr. A. E. Jacobson, NIADDK. Dextrorphan, leveorphanol, and ketocyclazocine were the gift of Dr. W. A. Klee of NIH. Naloxone, atropine, carbamylcholine, propranolol, dopamine, and serotonine were purchased from Sigma, St. Louis, MO.

Methods

Tissue Preparation. Synaptosomal membranes were prepared from rat hippocampi essentially as described by Zukin et al. (1974). Charles Rivers derived (CD) male rats (200–250 g) obtained from Levinstein's Farm (Yokenam, Israel) were decapitated and their hippocampi rapidly dissected out and homogenized in 20 volumes of ice-cold 0.32 M sucrose in a glass homogenizer fitted with a Teflon pestle. The sucrose solution, as well as the others solutions used during preparation of synaptosomes, contained the following antiproteases: 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 3 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 5 units/mL aprotinine, and 5 μg/mL pepstatin A. The homogenate was centrifuged at 1000*g* for 10 min, the pellet was discarded, and the supernatant was centrifuged at 20000*g* for 20 min. The pellet was resuspended in distilled water containing the above cocktail of antiproteases and dispersed with sonicator Model W-10 (Heat Systems-Ultrasonics Inc.) at setting 7 for 20 s. The suspension was centrifuged at 8000*g* for 20 min. The supernatant was collected and the upper coat of the pellet rinsed carefully with the supernatant fluid in order to collect the upper layer of the pellet. The combined supernatant was then centrifuged at 48000*g* for 20 min. The final crude synaptic membrane pellets were resuspended in 50 mM Tris-HCl buffer, pH 7.4, containing the cocktail of antiproteases and used immediately either for binding or for photoaffinity labeling experiments. We did not use frozen membranes since preliminary experiments had shown that after freezing–thawing the degree of photoaffinity labeling was lower than that in freshly prepared membranes. Binding of [^{3}H]PCP or [^{3}H]AZ-PCP, however, was not affected by freezing–thawing of the membranes. We also found that although antiproteases were not needed for the binding experiments, they were required for the photoaffinity labeling experiments since in their absence the extent of labeling was markedly reduced, possibly as a result of proteolytic degradation of labeled polypeptides.

Binding Assays. Aliquots of synaptosomal membranes (100–120 μg of protein) were incubated in 200 μL of 50 mM Tris-HCl buffer containing the indicated concentrations of [^{3}H]PCP or [^{3}H]AZ-PCP at 25 °C, for 15 min. Under these conditions, binding of the labeled ligands reached equilibrium; binding isotherms identical with those presented (see Figure 1) were observed when incubation periods were increased to 30 or to 60 min. Nonspecific binding was determined in samples containing the labeled ligands and 100 μM unlabeled PCP, and corrections for adsorption to filters were made as described (Hampton et al., 1982). In competition binding experiments the reaction mixtures contained 50 nM [^{3}H]PCP or 50 nM [^{3}H]AZ-PCP and various concentrations of the unlabeled competing ligand. Reactions were terminated by the addition of 3 mL of ice-cold buffer and rapid filtration (<10 s) on Whatman GF/C glass filters presoaked in 0.05% poly(ethylenimine), pH 7.4, as described by Hampton et al.

[1] Abbreviations: PCP, 1-(1-phenylcyclohexyl)piperidine (phencyclidine); TCP, *N*-[1-(2-thienyl)cyclohexyl]piperidine; hydroxy-PCP, *N*-[1-(3-hydroxyphenyl)cyclohexyl]piperidine; SKF 10047, *N*-allylnormetazocine; AZ-PCP, *N*-[1-(3-azidophenyl)cyclohexyl]piperidine; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

(1982). The filters were washed twice with 3 mL of ice-cold buffer and counted in 4 mL of scintillation liquid (Hydro-Luma, Lumac Systems). Nonspecific adsorbance of labeled drugs to the filters (in the absence of membranes) was low and was subtracted from total counts. All assays were carried out in triplicate together with triplicate samples containing 100 μM unlabeled PCP. Specific binding was calculated as the total minus the nonspecific binding. All binding experiments employing AZ-PCP or [^{3}H]AZ-PCP were carried out in the dark. Competition experiments with AZ-PCP (or PCP) and [^{3}H]morphine or [^{3}H]ethylketocyclazocine were performed as described by Itzhak et al. (1984).

Photoaffinity Labeling. Aliquots (200 μL) of synaptosomal membranes (200–250 μg of protein), freshly prepared in the antiprotease buffer, were incubated in 2.4 mL of 10 mM sodium phosphate buffer, pH 7.4, containing 100 nM [^{3}H]-AZ-PCP with or without the indicated concentrations of unlabeled drug, for 15 min at 25 °C in the dark. For nonspecific labeling 1 mM TCP was employed. Phosphate buffer was used since the efficiency of labeling was found to be greatly diminished when Tris-HCl buffer was used.

Following incubation in the dark, samples were withdrawn for determination of total binding. The reaction mixture was then photolyzed with a longwave 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. After photolysis, membranes were either precipitated and washed 4 times with Tris-HCl buffer (50 mM, pH 7.4) containing the cocktail of antiproteases or precipitated and diluted 500-fold in the same buffer with one addition of 1 mM unlabeled PCP and filtered on GE/C filters after 60 min of incubation at room temperature. The amount of specifically bound [^{3}H]AZ-PCP after precipitation and dilution was assumed to reflect the extent of the covalent binding of the ligand. This assumption was verified by means of an additional assay, in which photolabeled membranes were solubilized in 1% SDS solution. One milliliter of 0.1 g/mL activated charcoal and 20 mg/mL BSA in 10 mM sodium phosphate buffer, pH 7.4, were added to 200 μL of the solubilized membranes (containing ~200 μg of protein). After 15 min the mixture was centrifuged, and 100-μL samples from the supernatant were counted. The specific covalent labeling was calculated by subtracting the nonspecific labeling (determined in the presence of 1 mM TCP) from the total counts present in the supernatant. In the absence of membranes, photolyzed [^{3}H]AZ-PCP was adsorbed to the charcoal, by more than 97%. After precipitation and washing, the photoaffinity labeled membranes were used for gel electrophoresis.

Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970). Membrane samples (150–200 μg of protein) were dissolved 1:1 (v/v) in 2× sample buffer (52.5 mM Tris-HCl, pH 6.8, 10% glycerol, 5% 2-mercaptoethanol) and applied after ~2 h to polyacrylamide slab gels (10–12.5%). Proteins were electrophoresed together with molecular weight standards (Pharmacia Fine Chemicals, low molecular weight proteins) at 15–20 mA/slab for 3 h and then stained with 0.2% Coomassie Brilliant Blue in 50% methanol and 7% acetic acid and destained in 7.5% acetic acid. Gels were sliced into 2-mm sections with a gel slicer. Each slice was digested in 5 mL of Lipoluma–Lumasolve–Water (10:1:0.2) (Lumac Inc.) in a closed scintillation vial. Radioactivity was determined after 24 h by liquid scintillation spectrometry.

Two-dimensional gel electrophoresis was carried out essentially as described by Roberts et al. (1984). Membranes (1.2 mg) were dissolved in 0.5 mL of buffer containing 9.3 M urea, 5 mM K_2CO_3, and 0.2 mM PMSF and sonicated as described (Roberts et al., 1984). Nonidet P40 (10 μL) and dithiothreitol (2.5 mg) were then added, and the solution was centrifuged at 13000*g* for 20 min at 10 °C.

Ampholin solutions (1% pH 3.5–10.0 and 1% pH 5.0–8.0, LKB) were added to the collected supernatant. To the first dimensional tube gel prepared as described (Roberts et al., 1984), 150 μL (containing ~300 μg of protein) was applied and run at 300 V for 15 h followed by a run of 600 V for 2 h. Each run included a separate tube gel for measuring the pH gradient. The gels were then removed to SDS equilibrium buffer (Roberts et al., 1984) containing 1% 2-mercaptoethanol (v/v), incubated for 15 min, and subjected to second-dimension separation by SDS–polyacrylamide gel electrophoresis as described above. Gels were then stained, destained, sliced, and counted as described above.

Table I: Potencies of Various Drugs in Displacing Bound [^{3}H]PCP and [^{3}H]AZ-PCP in Rat Brain Hippocampus[a]

drug	inhibition of specific [^{3}H]PCP binding, I_{50} (μM)	inhibition of specific [^{3}H]AZ-PCP binding, I_{50} (μM)
PCP	0.3 ± 0.1 (0.17)	1.2 ± 0.24 (0.6)
AZ-PCP	0.5 ± 0.2 (0.25)	1.0 ± 0.15 (0.5)
OH-PCP	0.13 ± 0.04	0.22 ± 0.05
TCP	0.066 ± 0.02 (0.05)	0.08 ± 0.02 (0.07)
(+)-ketamine	1.7 ± 0.3 (1.3)	0.8 ± 0.15 (0.6)
(−)-ketamine	17.0 ± 5.0 (12.0)	6.0 ± 1.7 (5.0)
dexoxadrol	0.18 ± 0.08 (0.10)	0.1 ± 0.02 (0.07)
levoxadrol	43.0 ± 14.0 (29)	27.0 ± 8.5 (15)
dextrorphan	4.0 ± 1.0	7.0 ± 2.0
levorphanol	8.0 ± 1.7	10.0 ± 2.7
(±)-SKF 10047	1.5 ± 0.2 (0.7)	4.5 ± 1.8 (2.3)

[a] Inhibition of binding of [^{3}H]PCP and [^{3}H]AZ-PCP was determined in binding experiments as described under Methods and in Figure 2. I_{50} represents the drug concentration that results in 50% inhibition of the binding. Values are the mean ± SD of three separate determinations. Numbers in parentheses represent the I_{50} values determined in 10 mM sodium phosphate buffer.

RESULTS

Reversible Binding of [^{3}H]AZ-PCP to Rat Brain Hippocampus Membranes. The binding of AZ-PCP and of PCP was studied by employing an enriched synaptosomal fraction from rat hippocampus in 50 mM Tris-HCl buffer, pH 7.4, at 25 °C, using the rapid filtration assay (see Methods). Nonspecific binding of [^{3}H]PCP or of [^{3}H]AZ-PCP (determined in the presence of 100 μM PCP) was respectively 20 and 70% of the specific binding at 0.01 and 1.0 μM ligand concentrations. This binding was subtracted from the total binding. Typical binding isotherms for [^{3}H]AZ-PCP and [^{3}H]PCP are shown in Figure 1. For both ^{3}H-labeled ligands, specific and saturable binding is observed at the concentration range of 0.01–1.0 μM. Scatchard plots for [^{3}H]PCP and [^{3}H]AZ-PCP binding (Figure 1, inserts) are linear and extrapolate to the same B_{max} values (5.0 ± 1.0 pmol/mg of protein, n = 6, and 5.9 ± 0.6 pmol/mg of protein, n = 6, respectively). Their respective dissociation constants were 0.26 ± 0.05 and 0.25 ± 0.08 μM. Binding of the labeled ligands to the hippocampal synaptosomes was not affected by the presence of 10 μM morphine (Figure 1), suggesting that at the concentration range of 0.01–1.0 μM they bind mostly to the PCP receptor (Zukin & Zukin, 1979; Vincent et al., 1979) and not to the morphine (μ) receptors.

Additional support for this conclusion comes from displacement experiments (Figure 2, Table I). The binding of both [^{3}H]PCP (Figure 2A) and [^{3}H]AZ-PCP (Figure 2B) was

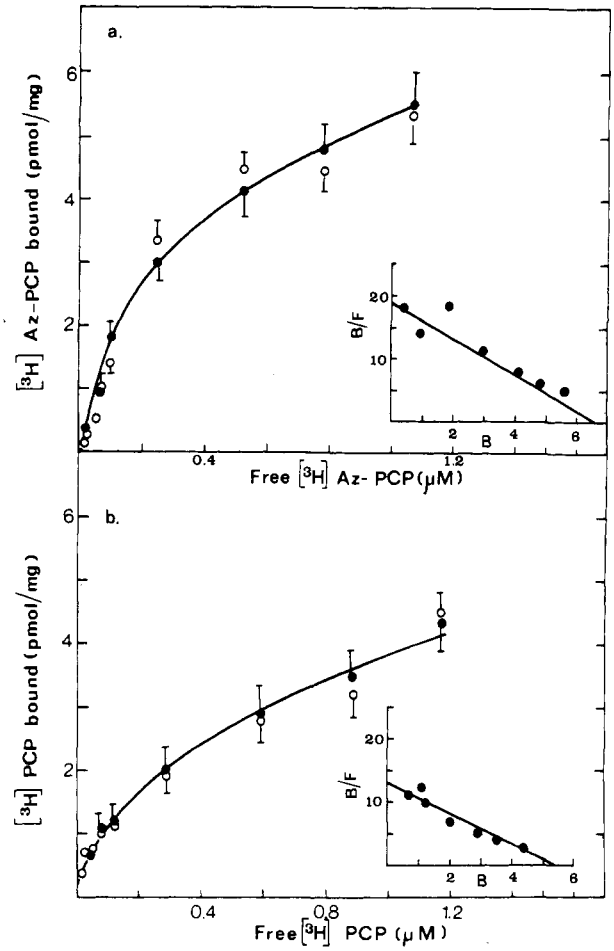

FIGURE 1: Specific binding of [^{3}H]PCP (a) and [^{3}H]AZ-PCP (b) to hippocampus synaptosomal preparation at 25 °C. Binding was assayed by the filtration method as described under Methods in the presence (○) or absence (●) of 10 μM morphine, as described under Methods. Each point is the mean value of three separate determinations. (Inserts) Scatchard plots of the binding data in the absence of morphine. B = specific bound ligand in pmol/mg of protein. B/F = bound over free ligand [pmol (mg of protein)$^{-1}$ M × 10^6]. Vertical bars represent SD.

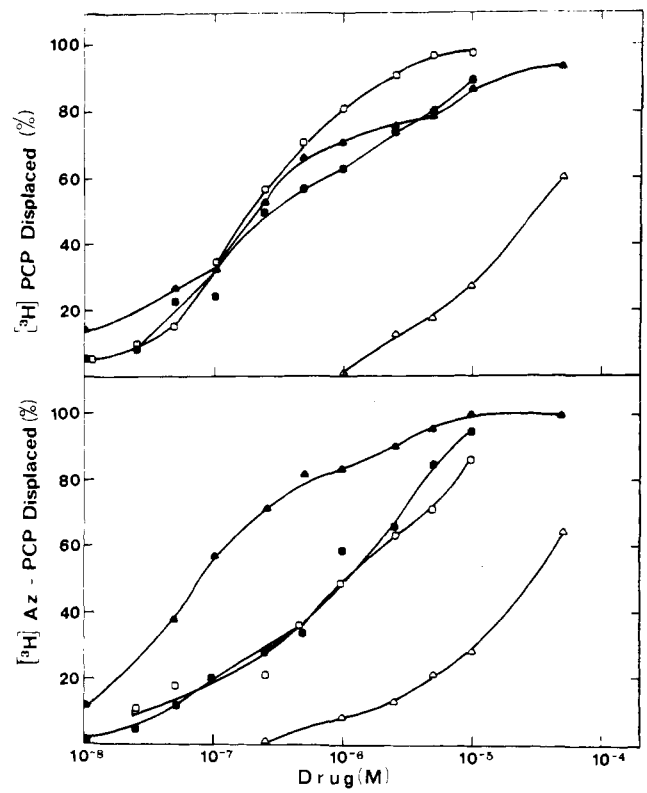

FIGURE 2: Inhibition of binding of [^{3}H]PCP and [^{3}H]AZ-PCP. Aliquots of membrane preparation were incubated with 50 nM ^{3}H-labeled ligand and with the unlabeled drugs at the concentrations indicated. Binding was determined (three separate determinations) by the filtration assay as described under Methods. Data are presented as percentage inhibition of the specific binding of ^{3}H-labeled ligand by PCP (□), AZ-PCP (■), dexoxadrol (▲), and levoxadrol (△).

displaceable by unlabeled PCP or AZ-PCP, as well as by dexoxadrol. The inactive isomer levoxadrol (Mendelsohn et al., 1984) was far less potent in displacing the labeled ligands (I_{50} > 10 μM, Figure 2). Similarly, the opioids naloxone, morphine, Leu-enkephalin, D-Ala-D-Leu-enkephalin, ethylketocyclazocine, and ketocyclazocine at 10 μM did not displace bound [^{3}H]AZ-PCP, while (±)-SKF 10047, TCP, (+)-ketamine, (−)-ketamine, hydroxy-PCP, dextrorphan, and levorphanol were all potent inhibitors of [^{3}H]AZ-PCP binding (Table I). The potency of the above drugs in displacing [^{3}H]PCP binding was similar to their potencies in displacing [^{3}H]AZ-PCP binding, and their I_{50} values are in close agreement with those reported previously (Zukin & Zukin, 1979; Vincent et al., 1979; Mendelsohn et al., 1984). Furthermore, in line with other studies, using [^{3}H]PCP (Vincent et al., 1979), atropine, carbachol, propranolol, dopamine, and serotonin at concentrations of 10 μM did not affect [^{3}H]AZ-PCP binding.

In subsequent photoaffinity labeling experiments, membranes were incubated in 10 mM sodium phosphate buffer, pH 7.4. Phosphate buffer was used since the efficiency of labeling was found to be greatly diminished when Tris-HCl buffer was used. In separate experiments we verified that the binding and specificity of [^{3}H]AZ-PCP and [^{3}H]PCP in 10 mM phosphate buffer are similar to those observed in 20 mM Tris-HCl buffer. Thus, the dissociation constants of [^{3}H]PCP and [^{3}H]AZ-PCP in 10 mM sodium phosphate buffer were 0.09 ± 0.02 and 0.12 ± 0.02 μM, respectively. The higher affinity of PCP and of PCP receptor ligands (Table I) in the phosphate buffer, as compared to that observed in the Tris-HCl buffer, is probably because of the differences in ionic strengths of the two buffers as described previously (Lazdunski et al., 1983).

Taken together, the data on reversible binding of [^{3}H]AZ-PCP indicate that this ligand binds with a specificity similar to that of [^{3}H]PCP to the PCP/σ receptors present in mammalian brain (Zukin et al., 1983; Itzhak et al., 1984) and is therefore a suitable ligand for the photoaffinity labeling of these receptors. As in the case of PCP (Itzhak et al., 1981), AZ-PCP displayed lower affinity toward morphine (μ) and κ receptors than toward the PCP/σ receptors; I_{50} values for AZ-PCP in displacing 1.0 nM [^{3}H]morphine and 2 nM [^{3}H]ethylketocyclazocine were 25 and 40 μM, respectively, as compared to 0.5 μM obtained for the displacement of 50 nM [^{3}H]PCP.

Photoaffinity Labeling of PCP Binding Sites. The photolabeling experiments were performed in three steps. First, the synaptosomal membranes were incubated for 15 min in the dark in 10 mM sodium phosphate buffer with [^{3}H]AZ-PCP under the conditions described above. Next, the reaction mixture was irradiated for 5 min with a long-wave UV lamp (see Methods). In the last step, the membranes were precipitated, the supernatant was discarded, and the pellets were resuspended either in ligand-free incubation buffer or in sample buffer for gel electrophoresis. Under these conditions it appeared to be possible to label the PCP binding sites without subjecting the PCP receptors to damage by the UV light. That

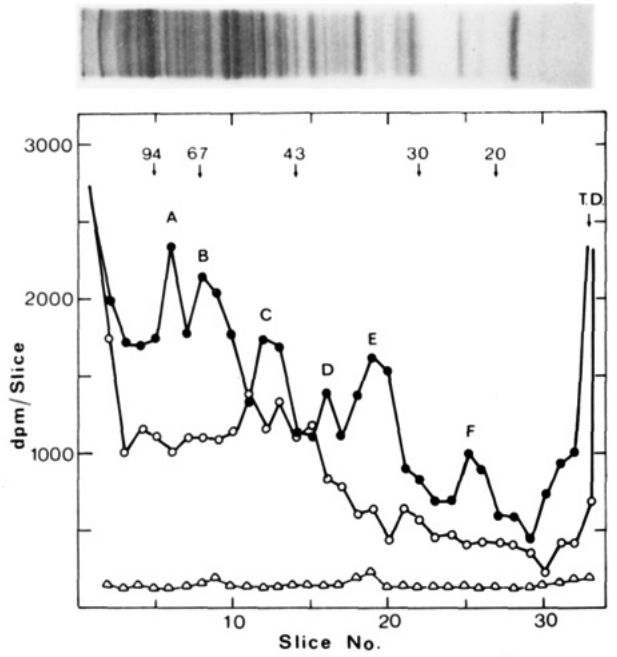

FIGURE 3: SDS–polyacrylamide gel electrophoresis of [^{3}H]AZ-PCP photolabeled hippocampus synaptosomal membranes. Membranes were incubated with 100 nM [^{3}H]AZ-PCP for 15 min in the dark and then UV irradiated for 5 min and electrophoresed. The gel was 12.5% acrylamid. (Upper) Coomassie Blue staining pattern of the gel. (Lower) Labeling pattern of the gel. Incubations were under the following conditions: irradiated membranes (●), nonirradiated membranes (△), and irradiated boiled membranes (○). In the latter, membranes were heated for 10 min at 100 °C and then used for the labeling experiments. Migration of molecular mass standards (in kilodaltons) in corun lanes is shown. T.D. is tracking dye.

is, the specific [^{3}H]PCP binding was not reduced in membranes exposed to UV light in the absence of ligand. Immediate filtration of the labeled membranes (50 μg of protein) yielded similar amounts of specific binding for both irradiated (3600 ± 200 cpm) and nonirradiated (3900 ± 180 cpm) membranes. In contrast, when the membranes were precipitated, diluted with a buffer containing 1 mM unlabeled PCP, and filtered after 60 min of incubation at 25 °C, only the irradiated membranes possessed irreversibly bound [^{3}H]AZ-PCP (1500 ± 100 cpm). The amount of irreversibly bound ligand corresponds to 30–40% of the reversible binding. Similar recoveries (~30%) of photolabeled [^{3}H]AZ-PCP receptor complexes were recorded in an additional assay in which the labeled membranes were first solubilized with 1% SDS and specific labeling was then determined with activated charcoal, which adsorbs the free ligand (see Methods).

Polyacrylamide–SDS gel electrophoresis was carried out in order to separate and identify the [^{3}H]AZ-PCP photoaffinity labeled components of the hippocampus synaptosomes. A typical labeling pattern (see Figure 3) shows five distinct radiolabeled peaks, corresponding to polypeptides with apparent M_r of 90 000 ± 1000 (peak A), 62 000 ± 4000 (peak B), 49 000 ± 2000 (peak C), 40 000 ± 1000 (peak D), and 33 000 ± 1000 (peak E). Each of these apparent molecular weights represents the mean ± SD of 14 separate determinations, using both 10% acrylamide and 12.5% acrylamide gels. Some experiments yielded additional peaks at lower molecular weights (M_r 24 000 and 17 000) (e.g., peak F in Figure 3), but the above five radiolabeled peaks appeared consistently in all experiments. A further series of experiments enabled us to conclude that the observed radiolabeled peaks correspond to [^{3}H]AZ-PCP photolabeled components of the intact membranes: the peaks did not appear when photoaffinity labeling

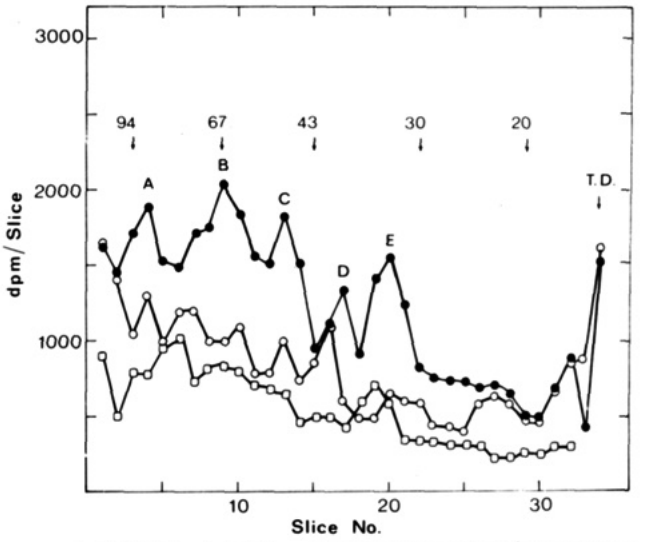

FIGURE 4: Inhibition by TCP and (±)-SKF 10047 of [^{3}H]AZ-PCP photoaffinity labeling. Hippocampal synaptosomal membranes were incubated in the dark for 15 min with 100 nM [^{3}H]AZ-PCP in the absence (●) and in the presence of 1 mM (±)-SKF 10047 (○) or 1 mM TCP (□). Membranes were then irradiated, subjected to SDS gel electrophoresis, electrophoresed, and processed for counting as described in Figure 3. The gel was 12.5% acrylamide. Each lane was loaded with 150 μg of protein. Migration of molecular mass standards (in kilodaltons) in corun lanes is shown. T.D. is tracking dye.

Table II: Inhibition of [^{3}H]AZ-PCP-Labeled Polypeptides by PCP Analogues and Opiate Receptor Ligands[a]

drug	% inhibition of specific labeling for peak				
	A	B	C	D	E
hydroxy–PCP	90 ± 5	92 ± 4	95 ± 1	95 ± 3	96 ± 2
TCP	96 ± 2	59 ± 2	59 ± 1	48 ± 7	33 ± 5
PCP	78 ± 4	44 ± 5	42 ± 6	29 ± 1	36 ± 2
(±)-SKF 10047	41 ± 5	26 ± 1	25 ± 3	22 ± 5	85 ± 6
dexoxadrol	78 ± 3	18 ± 3	22 ± 5	30 ± 4	70 ± 5
levoxadrol	5 ± 1	6 ± 3	6 ± 3	15 ± 3	9 ± 4
naloxone	4 ± 2	34 ± 6	10 ± 4	3 ± 3	7 ± 1
morphine	7 ± 3	27 ± 5	18 ± 5	5 ± 3	8 ± 3
D-Ala-D-Leu-enkephalin	4 ± 1	3 ± 1	9 ± 3	5 ± 1	2 ± 1

[a] Hippocampus synaptosomal membranes were photolabeled with 100 nM [^{3}H]AZ-PCP in the absence and in the presence of 10 μM of the indicated drug. The labeled membranes were then subjected to SDS gel electrophoresis. Gels were electrophoresed, sliced, and counted as described under Methods. Specific labeling present in each peak (as shown in Figures 2 and 3) was calculated by subtracting the counts measured in the presence of 1 mM TCP (nonspecific labeling) from the counts measured in the absence of unlabeled drugs, or from the counts measured in the presence of the various inhibitors. The former (dpm_1) reflects total specific labeling of each peak and the latter (dpm_2) their specific labeling in the presence of the inhibitors. Percent inhibition of specific labeling is then $100 - [[(dpm_1 - dpm_2) \times 100]/dpm_1]$. Values presented are the mean ± SD of three to four separate determinations.

was performed with boiled membranes (10 min, 100 °C) and could not be detected in gels of nonirradiated membranes (Figure 3).

In order to define the ligand specificity of the labeled components, we employed several PCP analogues and σ-opiate ligands. As shown in Figure 4, [^{3}H]AZ-PCP labeling was strongly inhibited by 1 mM TPC or 1 mM (±)-SKF. Similar inhibition was obtained with hydroxy-PCP and PCP. Specificity and selectivity were observed when lower drug concentrations were used (Table II). Hydroxy-PCP was found to be the most potent inhibitor of [^{3}H]AZ-PCP labeling, although it was not selective: at 10 μM it inhibited the labeling of all radioactive peaks (Table II). TCP and PCP were more selective: at 10 μM, both of these drugs strongly inhibited

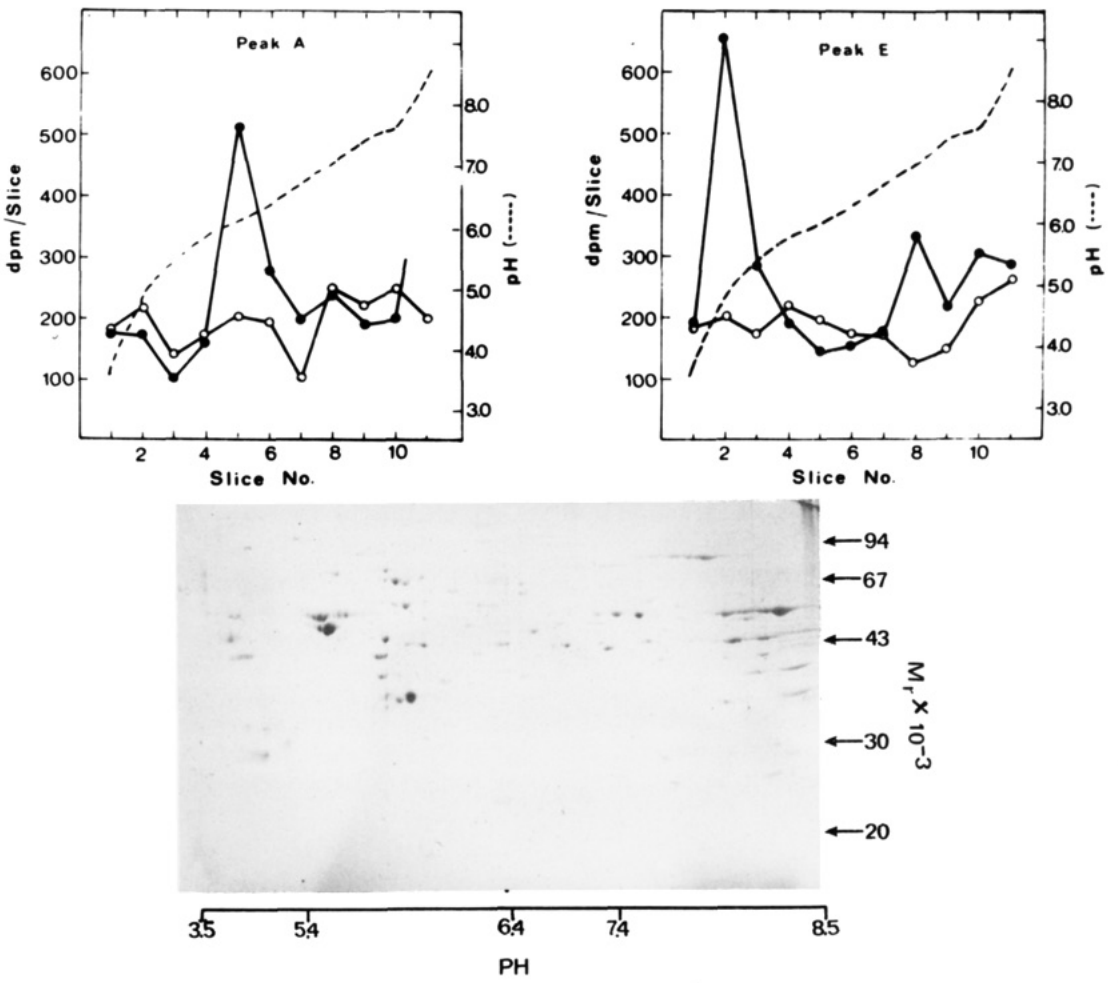

FIGURE 5: Two-dimensional gel electrophoresis of [^{3}H]AZ-PCP photolabeled hippocampus membranal proteins. Membranes were incubated and irradiated as described in Figure 3, in the presence (○) and in the absence (●) of 1 mM TCP. Membranes from each incubation (320 μg of protein) were processed for two-dimensional gel electrophoreses as described under Methods and electrophoresed in separate tubes (first dimension) along with molecular weight standards. Gels were stained and destained (lower) and lanes (6 mm) corresponding to molecular migration of M_r 90 000 (peak A) and ca. M_r 33 000 (peak E) (see Coomassie Blue staining pattern in the lower figure) were cut out from each gel. Note that these lanes are parallel to the pH gradient of the first dimension. Each lane was then sliced (1 cm) and counted as described under Methods (upper).

labeling of peak A and to a lesser extent of the other bands. TCP and PCP were more selective: at 10 μM, both of these drugs strongly inhibited labeling of peak A and to a lesser extent of the other bands. Note that peak E was inhibited the least by these two drugs, although it was strongly inhibited by (±)-SKF and dexoxadrol at 10 μM. Apparently, the latter two drugs inhibited selectively the labeling of peaks A and E (Table II).

The morphine receptor (μ) related drugs naloxone and morphine (Martin et al., 1976) and the δ-opioid receptor (Lord et al., 1977) related peptide D-Ala-D-Leu-enkephalin, all at 10 μM, did not inhibit the labeling of peaks A and E by [^{3}H]AZ-PCP. Apparently, except for morphine and naloxone, which inhibited peaks B and C to some extent, these drugs did not inhibit the labeling at all (Table II). The muscarinic antagonist atropine and the β receptor antagonists propronalol and serotonin, all at 10 μM, were also ineffective as inhibitors. Taken together, these results point to the possibility that among the various labeled polypeptides those of M_r 90 000 and possibly also of M_r 33 000 (peaks A and E) could represent components of the PCP/σ receptors. This suggestion is supported by data obtained in experiments using dexoxadrol and its inactive stereoisomer levoxadrol (Hampton et al., 1982; Mendelsohn et al., 1984; see also Table I): as shown in Table II, 10 μM dexoxadrol but not levoxadrol strongly inhibited the labeling of peaks A and E. Stereospecific labeling of these two bands is demonstrated in Table II.

Since the bands for both peak A and peak E on the SDS gels are broad, we had to consider the possibility that each of them contains more than one labeled component. We therefore performed two-dimensional gel electrophoresis (Roberts et al., 1984; see Methods) in order to separate the polypeptides that had undergone photoaffinity labeling by [^{3}H]AZ-PCP. After completion of the run, the gels were stained, and the areas corresponding to M_r of 90 000 (peak A) and M_r of 33 000 (peak E) were identified with molecular weight standards. Lanes corresponding to peaks A and E were cut, sliced, and subjected to radioactivity determination as described under Methods. Results of a typical experiment (shown in Figure 5) indicate that peak A corresponds mainly to a single polypeptide focused at pH 6.0–6.3. A major labeled polypeptide of peak E was focused at pH 4.6–5.4, and a minor component was detected at pH 7.0.

Discussion

The present study describes the reversible binding properties of [^{3}H]AZ-PCP to rat hippocampus membranes and the photoaffinity labeling of membranal proteins by this photoactivatable analogue of PCP. The reversible binding properties of [^{3}H]AZ-PCP are very similar to those of [^{3}H]PCP;

both ligands display relatively high affinity ($K_d \simeq 0.2$ μM) to an equal number of binding sites, the binding of each ^{3}H-labeled ligand is displaced by unlabeled PCP or AZ-PCP, and the pharmacological profile and stereospecificity of sites labeled by [^{3}H]AZ-PCP are identical with those of sites labeled by [^{3}H]PCP (Table I). The I_{50} values for various PCP analogues and σ-opiate ligands, determined in this study employing [^{3}H]AZ-PCP, are in agreement with their I_{50} values determined in earlier displacement experiments with [^{3}H]PCP (Quirion et al., 1981a; Hampton et al., 1982; Mendelsohn et al., 1984; Zukin et al., 1984). We therefore conclude that [^{3}H]AZ-PCP binds (like PCP) to a brain PCP receptor with binding characteristics similar to those of the σ receptor. It should be noted that although our direct binding data with [^{3}H]PCP and [^{3}H]AZ-PCP yield linear Scatchard plots, the competition binding curves [e.g., for dexoxadrol and (±)-SKF 10047] span over a concentration range of more than 3 orders of magnitude. Thus, it is possible that [^{3}H]PCP and [^{3}H]-AZ-PCP each binds to more than one class of binding sites, suggesting that the PCP receptors are heterogeneous. Interestingly, insertion of the azido moiety into the phenyl ring did not change the binding selectivity of the ligand either to the PCP receptors ($K_d \simeq 0.2$ μM) or to μ and κ receptors ($I_{50} >$ 20 μM). This is in contrast to other substitutions and modifications of the PCP molecule, which have led to dramatic changes in the potency and selectivity of the analogue. For example, hydroxy-PCP was shown to bind with higher affinity than PCP to the PCP receptor (Vignon et al., 1982) and (unlike PCP) to bind with high affinity to the μ receptors (Itzhak et al., 1981). The analogue TCP was shown to bind selectively to the PCP/σ receptors (Vignon et al., 1983; Lazdunski et al., 1983). Substitution of hydrogens at position 4 of the cyclohexyl ring of PCP by hydroxy and phenyl residues yielded a selective μ ligand (Itzhak & Simon, 1984).

Thus, reversible binding data of [^{3}H]AZ-PCP indicate that the ligand is suitable for labeling of brain PCP receptors. In view of the high affinity of [^{3}H]AZ-PCP to the PCP receptors (0.2 μM) relative to the μ and κ receptors ($I_{50} >$ 20 μM), it seems likely that at the submicromolar concentration range the drug would bind to the PCP receptor and not to the ligand binding sites of opiate receptors. Accordingly, in our photolabeling experiments with [^{3}H]AZ-PCP we used concentrations of 70–100 nM. Irradiation of the [^{3}H]AZ-PCP–receptor complexes consistently yielded five major labeled polypeptides. Among the various labeled bands two displayed stereospecific labeling, namely, those corresponding to polypeptide with apparent M_r of 90 000 and 33 000 (peaks A and E, Figure 3). Labeling of these bands underwent varying degrees of inhibition by 10 μM PCP analogues and (±)-SKF 10047 but not by 10 μM naloxone, morphine, or D-Ala-D-Leu-enkephaline, when these were present during incubation and photolabeling. Thus, the pharmacological profile of the M_r 90 000 and of the M_r 33 000 polypeptides labeled by [^{3}H]AZ-PCP closely resembles the profile of the PCP/σ receptor. It is tempting to propose that these polypeptides are components of the PCP/σ receptor.

Although labeling of the M_r 90 000 and the M_r 33 000 bands was highly reproducible, the above suggestion must be treated with some caution. One cannot rule out the possible occurrence of proteolysis during the experiment, although the inclusion of a cocktail of antiproteases in the reaction mixture during membrane preparation and incubation should have prevented proteolytic degradation—provided that all proteases present were sensitive to the inhibitors used. Since the two bands appear also after two-dimensional gel electrophoresis, it seems unlikely that proteolysis would have been caused during the run by an SDS-resistant protease that would have migrated together with the labeled polypeptides in the SDS–polyacrylamide gel.

An important point that has to be accounted for is the different potencies of the various drugs in their inhibition of the labeling of the M_r 90 000 and the M_r 33 000 polypeptides. Labeling of both polypeptides is equally sensitive to hydroxy-PCP and dexoxadrol. We found, however, that the M_r 90 000 polypeptide is highly sensitive to TCP and PCP and less sensitive to (±)-SKF 10047, whereas the M_r 33 000 band is highly sensitive to (±)-SKF 10047 and less sensitive to TCP and PCP (Table II). The lower sensitivity of the M_r 90 000 polypeptide to (±)-SKF as compared to its sensitivity to TCP, hydroxy-PCP, and PCP is reminiscent of the lower affinity of the former drug toward the PCP receptor (Zukin et al., 1984; Hampton et al., 1982; Table I). This, together with the stereospecific labeling of the M_r 90 000 polypeptide and its appearance as a single peak even after two-dimensional electrophoresis, would be in line with the hypothesis that this polypeptide represents a component of the PCP/σ receptor.

The [^{3}H]AZ-PCP-labeled band of M_r 33 000, unlike the M_r 90 000 band, appears to be composed of two distinct polypeptides (Figure 5). In preliminary experiments using autoradiography, we have observed that the M_r 33 000 band appears as a doublet in the SDS–polyacrylamide slab gels, suggesting that the two polypeptides may have slightly different molecular weights. The pharmacological profile of the two bands has not yet been characterized. It is possible, however, that one of the M_r 33 000 polypeptides is a component of the PCP/σ receptor and that the other is related to a different class of sites. This hypothesis could explain why the more selective PCP/σ receptor ligands TCP and PCP (Vignon et al., 1983; Lazdunski et al., 1983) only partially inhibited the labeling of the M_r 33 000 bands, whereas hydroxy-PCP and (±)-SKF 10047, which are less selective ligands (Itzhak et al., 1981; Zukin & Zukin, 1981), strongly inhibited labeling of this band: i.e., the two latter ligands may have inhibited the labeling of two polypeptides of M_r 33 000, one of which is not related to the PCP/σ receptor.

Apart from the two labeled bands discussed above, the [^{3}H]AZ-PCP labeled three more bands. The labeling of these latter bands (M_r of 62 000, 49 000, and 40 000) was specific as determined by the inhibition of their labeling by PCP analogues (Table II) and by the failure of various opiate, muscarinic, and β-adrenergic ligands to eliminate the labeling. However, the pharmacological characteristics of these labeled bands are not as clear as those of the M_r 90 000 and 33 000 polypeptides that resemble the PCP/σ receptor. Thus, the inhibition of the labeling of the M_r 62 000, 49 000, and 40 000 bands by (±)-SKF 10047 and by dexoxadrol is relatively low (Table II). Moreover, the labeling of the M_r 62 000 and 49 000 bands was inhibited by 10 μM morphine (which does not inhibit PCP and AZ-PCP binding) to the same extent as that by (±)-SKF 10047 and dexoxadrol (20–30% reduction in the labeling). These differences could indicate that the sites on the M_r 62 000, 49 000, and 40 000 bands, which are labeled by [^{3}H]AZ-PCP, are distinct from the major recognition site of PCP/σ receptor.

Alternatively, the labeled bands that share lower PCP/σ receptor specificity could be constituents of a second PCP receptor with somewhat different characteristics than the site containing the M_r 90 000 and M_r 33 000 bands. For example, as specific interactions of both PCP (Blaustein & Itzkovicz, 1983; Albuquerque et al., 1981) and AZ-PCP (Sorensen &

Blaustein, 1984) with presynaptic K channel have been demonstrated, it is possible that M_r 62000, 49000, and 40000 bands are constituents of a potassium channel. It should be noted that both of the above alternatives are consistent with the present competition binding data, which suggest heterogeneity of the PCP binding sites. However, we cannot completely rule out the possibility that in spite of the inhibition by PCP analogues these bands represent some type of a nonspecific interaction.

It is interesting to note that Sorensen & Blaustein (1984) have reported a preliminary study on the photoaffinity labeling of rat brain membranes with [^{3}H]AZ-PCP. Although the sensitivity of labeling to various pharmacological active drugs was not reported, these authors indicated that several polypeptides were labeled by [^{3}H]AZ-PCP, in agreement with the results reported here. In this connection, it should be noted that photoaffinity labeling of other receptors (e.g., nicotinic cholinergic receptors or opioid receptors) have also yielded labeling of multiple bands (Oswald & Changeux, 1981; Haring et al., 1983a, 1984; Bidlack et al., 1981), which in the case of the nicotinic receptors are constituents of the receptor molecule. Interestingly, polypeptides with molecular weights similar to those labeled by [^{3}H]AZ-PCP were also identified by affinity labeling or affinity chromatography of opioid receptors. These include bands of M_r 35000 (bidlack et al., 1981; Zukin & Kream, 1979; Maneckjee et al., 1985), M_r 44000 (Bidlack et al., 1981; Maneckjee et al., 1985), M_r 58000–60000 (Klee et al., 1982; Newman & Barnard, 1984), and M_r 94000 (Maneckjee et al., 1985). In view of the known ability of opiate ligands (including PCP analogues), to cross-react with the different classes of opioid receptors, it is possible that the various polypeptides labeled by [^{3}H]AZ-PCP are homologous to the components of opioid receptors. This intriguing hypothesis would be in line with the proposal that the PCP receptors are identical with the σ receptor sites (Quirion et al., 1981a; Zukin et al., 1984). Obviously, more data on the structure of nondenatured PCP receptors are needed to substantiate this hypothesis.

Registry No. PCP, 77-10-1; AZ-PCP, 90846-43-8.

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Four- and Five-Coordinate Species in Nickel-Reconstituted Hemoglobin and Myoglobin: Raman Identification of the Nickel-Histidine Stretching Mode†

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ABSTRACT: Nickel(II)-reconstituted hemoglobin (^{Ni}Hb) and myoglobin (^{Ni}Mb) and model Ni porphyrins have been investigated by Soret-resonance Raman difference spectroscopy. Two sets of frequencies for the oxidation-state and core-size marker lines in the region from 1300 to 1700 cm^{-1} indicate two distinct sites in ^{Ni}Hb. Only one of these sites is evident in the Raman spectra of ^{Ni}Mb. This result is consistent with the UV-visible absorption spectrum of ^{Ni}Hb, which shows two Soret bands at 397 and 420 nm and one Soret at 424 nm for ^{Ni}Mb. Excitation at the blue Soret component of ^{Ni}Hb with 406.7-nm laser radiation preferentially enhances the set of Raman marker lines typical of Ni-protoporphyrin IX [Ni(ProtoP)] in noncoordinating solvents. The wavelength of the blue Soret component and the Raman spectrum indicate four-coordination for this site in ^{Ni}Hb. Laser excitation in the red Soret band enhances a set of lines whose frequencies are compatible with neither four- nor six-coordinate frequencies but are intermediate between the two. The red Soret band of the proteins is also considerably less red shifted than six-coordinate Ni-porphyrin models. These results suggest that Ni in the second site possesses a single axial ligand. Raman spectra of ^{64}Ni-reconstituted and natural abundance Ni-reconstituted hemoglobins, obtained simultaneously in a Raman difference spectrometer, have identified the Ni-ligand stretch at 236 cm^{-1}. The line shifts to 229 cm^{-1} for the ^{64}Ni-reconstituted Hb. For a pure Ni-ligand stretch a 10-cm^{-1} shift would be predicted. A decrease of almost 7 cm^{-1} indicates a high degree of Ni-ligand character for the assigned mode. The frequency of the Ni-ligand stretch, falling as it does in the range of Fe-histidine stretching frequencies, points to histidine as the axial fifth ligand. The frequency of the Ni-histidine mode is slightly higher than that for the Fe-histidine mode in hemoglobin but lower than that observed for horseradish peroxidase. We find that the Ni-histidine mode is 5 cm^{-1} higher in ^{Ni}Mb (R-like) than in ^{Ni}Hb (T structure). A similar increase in the Fe-histidine frequency is noted in the comparison of the native deoxy proteins. The Raman results show that the local structural changes associated with a change in quaternary structure are the same at the metal site for both Ni-reconstituted and Fe hemoglobins.

Catalysis, ligand binding, redox reactions, and light to chemical energy transduction are among the important functions of metalloporphyrin-containing proteins in biology. Analogous biomimetic chemistries are also of current interest. To further develop and understand these chemistries, a detailed understanding of the relationship between protein structure and metalloporphyrin reactivity for this class of proteins would be useful.

One method of investigating structure-reactivity relationships is by making a comparison of systematically modified enzymes. Recently, a variety of transition metals have been substituted for iron in the central core of the porphyrin ring

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