3640

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High-Affinity (^{3}H)-Dexoxadrol Binding to Rat Brain Membranes

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ABSTRACT

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Specific binding of (^{3}H)-dexoxadrol to rat brain membrane was saturable. The specific binding represented 74 to 82% of total binding. Scatchard analysis of the data demonstrated the existence of high- and low-affinity binding sites. The kinetic constants for the corresponding binding sites were as follows: Kd_1 = 1.68 nM, $Bmax_1$ = 0.4 pmol/mg protein, Kd_2 = 26.1 nM, and $Bmax_2$ = 3.7 pmol/mg protein. Binding reached equilibrium within 5 minutes of incubation at 25°C. Optimum binding was observed between the pH range of 7.4 to 9. Binding was dependent on protein concentration and was reduced by denaturation of protein and by proteolytic enzyme. (^{3}H)-dexoxadrol binding was inhibited by drugs pharmacologically similar to phencyclidine (PCP) and bound (^{3}H)-dexoxadrol could be rapidly displaced by cold PCP.

Key words: (^{3}H)-dexoxadrol, high affinity, binding sites

INTRODUCTION

In the last 5 years, several investigators have demonstrated the presence of specific PCP binding sites, both in the brain and peripheral tissues [Vincent et al., 1979; Vignon et al., 1982; Zukin and Zukin, 1979; Zukin et al., 1983; Hampton et al., 1982; Eldefrawi et al., 1982]. Slices of cerebral cortex that were incubated with ^{3}H-PCP and processed for autoradiography revealed the existence of a high-affinity PCP receptor in this area of the brain [Quirion et al., 1981a]. However, some of the earlier findings [Vincent et al., 1979; Zukin and Zukin, 1979] have been disputed because of the possibility of the artifact introduced by the use of glass fiber filter paper in the rapid filtration assay [Domino, 1981]. Indeed, (^{3}H)-PCP binds to filter paper and this bound (^{3}H)-PCP can be displaced by cold PCP and its analogs [Maayani and Weinstein, 1980]. Recently attempts have been made to decrease the binding of (^{3}H)-PCP

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to glass fiber filter paper by presoaking the filter paper either in a 0.05% aqueous solution of polyethyleneimine [Hampton et al., 1982], in 1% Prosil [Eldefrawi et al., 1982], or in 0.01% polylysine [Zukin et al., 1983]. With these modifications in the binding assay, the maximum number of binding sites (Bmax) of (^{3}H)-PCP has been about 2–3.5 pmol/mg protein (or approximately 200 to 350 pmol/g) in the brain [Vignon et al., 1982; Zukin et al., 1983] and 8 pmol/mg protein in crayfish muscle membrane [Eldefrawi et al., 1982]. The Bmax for phencyclidine receptors in the brain is high relative to that of other receptors that have been characterized in this tissue. The Bmax (pmol/g) of some of the receptors is as follows: presynaptic alpha-adrenergic, 12 [U'Prichard and Snyder, 1979]; benzodiazepine, 18 [Squires and Braestrup, 1977]; serotonin, 20 [Bennett and Snyder, 1976]; opiate, 30 [Pert and Snyder, 1983]; and muscarinic cholinergic, 65 [Yamamura and Snyder, 1974]. The scientific literature indicates that the problems of the (^{3}H)-PCP binding assay have not yet been resolved. Therefore an attempt has been made to use a biolog of PCP as a probe. We have assumed that this biolog can label the PCP binding sites. The results of our investigations with the PCP biolog (^{3}H)-dexoxadrol are described in this report.

MATERIALS AND METHODS

Chemical and Drugs

(^{3}H)-Dexoxadrol was prepared by the catalytic reduction with tritium. The product was purified by thin layer chromatography and was 95% radiochemically pure by high pressure liquid chromatography. The specific activity of (^{3}H)-dexoxadrol was 47.6 Ci/mmol. Trizma base, chymopapain, proteinase K, thermolysin, trypsin, 2-mercaptoethanol, and p-chloromercuriphenyl sulfonic acid (PCMPSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Dimethylphenylpiperazinium (DMPP) was obtained from Aldrich Chemical Co. Phencyclidine, dexoxadrol, levoxadrol, and etoxadrol were synthesized at the Upjohn Company. (+)1-(1-Phenylcyclohexyl)-3-methyl-piperidine ((+)PMCP) and (−)PCMP were generous gifts from Dr. Kenner C. Rice of the National Institute of Mental Health. (±)Cyclazocine, (±)SKF 10047, their respective isomers, and LSD were kindly provided by Dr. R. Willette of the National Institute of Drug Abuse.

Membrane Preparation

Male Sprague-Dawley rats (180–250 g) were sacrificed by decapitation and the brains were quickly removed from the skull. Discrete brain areas [dissected by the method of Glowinski and Iversen, 1966], or whole brain minus cerebellum, were placed in 10 volumes of cold (4°C) 50 mM Tris-HCl buffer, pH 7.4, and homogenized using a Brinkman Polytron PCU 2-11 homogenizer for 30 seconds at setting #6, and centrifuged at 1,000 × g for 10 minutes. The supernatant was centrifuged at 48,000 × g for 20 minutes and the resulting pellet was washed again by resuspension and recentrifugation at 48,000 × g for 20 minutes. The final pellet was resuspended in 80 volumes (w/v) of the same buffer.

Binding Assay

(^{3}H) Dexoxadrol binding was measured by incubating 0.8 ml aliquots of tissue suspension in triplicate with 0.1 ml of (^{3}H) dexoxadrol to give a final concentration in the range of 0.1 to 22 nM, and 0.1 ml of distilled water or drug, to give a final volume of 1.0 ml. This mixture was incubated for 30 minutes at 25°C and then filtered under vacuum through a Whatman GF/B filter. The incubation tube was rinsed with 5 ml cold (4°C) buffer and this rinse was applied to the filter. The filter was finally washed three times with 5-ml aliquots of buffer. The filter paper was placed in a scintillation vial to which 15 ml of Amersham Searle AC'S cocktail was added. The vials were shaken on an Eberbach mechanical shaker for 30

minutes and then the radioactivity was counted by a liquid scintillation spectrometer. Proteins were determined by the method of Lowry et al. [1951] using bovine serum albumin as a standard.

Specific binding was defined as the total binding minus binding in the presence of 1 mM phencyclidine. Specific binding represented 74 to 82% of the total binding.

Time Course of (^{3}H)-Dexoxadrol Binding

To determine an appropriate incubation time, the binding of (^{3}H)-dexoxadrol to whole rat brain (1:80 w/v) was measured as a function of time in three independent experiments. Membrane preparation was added to initiate binding, and the reaction was terminated at a designated time by filtration of the sample under vacuum. The specific binding was determined as indicated above.

Effect of pH on (^{3}H)-Dexoxadrol Binding

The pH of 50 mM Trizma base was adjusted to the designated pH (3.0 to 9.0) by using 1 to 12.1N HCl. The final pellet was suspended in 80 volumes (w/v) of the indicated Tris-HCl buffer. The binding assays were carried out as described above.

Effect of Protein Concentration and Protein Denaturation on (^{3}H)-Dexoxadrol Binding

The final pellet of whole rat brain was suspended in 10, 20, 40, 60, 80, 100, 120, and 160 volumes (w/v) of 50 mM Tris-HCl buffer, pH 7.4. These membrane preparations were used for binding assays as mentioned above. For denaturation of protein, a tissue sample, 1:80 (w/v), was boiled for 30 minutes and then placed on ice for 30 minutes prior to its use in the binding assay.

Effect of Proteolytic Enzymes and Sulfhydryl Group Inhibitors on (^{3}H)-Dexoxadrol Binding

Aliquots of the membrane suspension (1:80 w/v) of whole rat brain were preincubated for 10 minutes at 25°C with an appropriate concentration (Table 2) of proteolytic enzymes. Reaction was terminated by placing the samples on ice. The samples were then used in (^{3}H)-dexoxadrol binding assays. An experiment with sulfhydryl group inhibitors involved two 10 minute preincubations. First the samples were incubated with 1 mM 2-mercaptoethanol or its vehicle (Tris-HCl buffer). Then PCMPSA, a sulfhydryl group inhibitor (final concentration 1 mM), or its vehicle (D/W) was added to the designated sample. The reaction was terminated by placing the samples on ice. The samples were then assayed using (^{3}H)-dexoxadrol binding.

Displacement of Bound (^{3}H)-Dexoxadrol by 100 μM PCP

Aliquots of the membrane suspension (1:80 w/v) were incubated with (^{3}H)-dexoxadrol (2 nM) for 30 minutes at 25°C. At 30 minutes, PCP was added (final concentration 100 μm) and the reaction was terminated at various time intervals after the addition of PCP. Specific binding was determined as indicated above.

Analysis of Data

Data were subjected to Scatchard analysis. Dissociation constants were determined by linear regression analysis and Bmax was calculated by the following equation: $Y = a+bx$, where a is the intercept on the x-axis at $Y = 0$, and b is the slope. IC_{50} values were obtained from logit-log plot of the data. Inhibition constants (Ki) were calculated by the equation: $Ki = IC_{50}/1 + (C/Kd_1)$, where C is the concentration of ligand and Kd_1 is the dissociation constant of high-affinity binding sites.

RESULTS

The Specific Binding of (^{3}H)-Dexoxadrol to Whole Rat Brain Membranes

The specific binding of (^{3}H)-dexoxadrol was saturable with respect to ligand concentration (Fig. 1 inset) and it represented 74 to 82% of the total binding. A Scatchard plot (Fig. 1) of (^{3}H)-dexoxadrol binding to whole rat brain suspension was hyperbolic with concavity upward, and could be resolved into two linear components. These two linear components suggested the existence of high- and low-affinity (^{3}H)-dexoxadrol binding sites whose dissociation constants differed by a factor of 15.5. The high-affinity component had a dissociation constant (Kd_1) of 1.68 ± 0.2 nM (n = 7), with a $Bmax_1$ of 0.4 ± 0.06 pmol/mg protein (n = 7) that is approximately 9.6% of the total binding. The corresponding values for the low-affinity components were Kd_2 = 26.1 ± 2.5 nM and $Bmax_2$ = 3.7 ± 0.2 pmol/mg protein for n = 7. (^{3}H)-Dexoxadrol bound rapidly to its high-affinity binding site (Fig. 2). Within 1 minute, 74.5% of specific binding sites were occupied by (^{3}H)-dexoxadrol. There was a small but significant increase in binding over the next 59 mintues.

Effect of pH on (^{3}H)-Dexoxadrol Binding

The binding of (^{3}H)-dexoxadrol as a function of pH was investigated at a pH range of 3 to 9 (Fig. 3). Nonspecific binding to both filters and membranes was relatively high at pH below 7.4. At pH 7.4, there was a significant reduction in nonspecific binding and it was maintained at that level at pH 8, 8.5, and 9. The specific binding reached optimum between the pH of 7.4 and 8.5. All the investigations reported in the present study were carried out at pH 7.4 because that is the physiological pH of biological fluids.

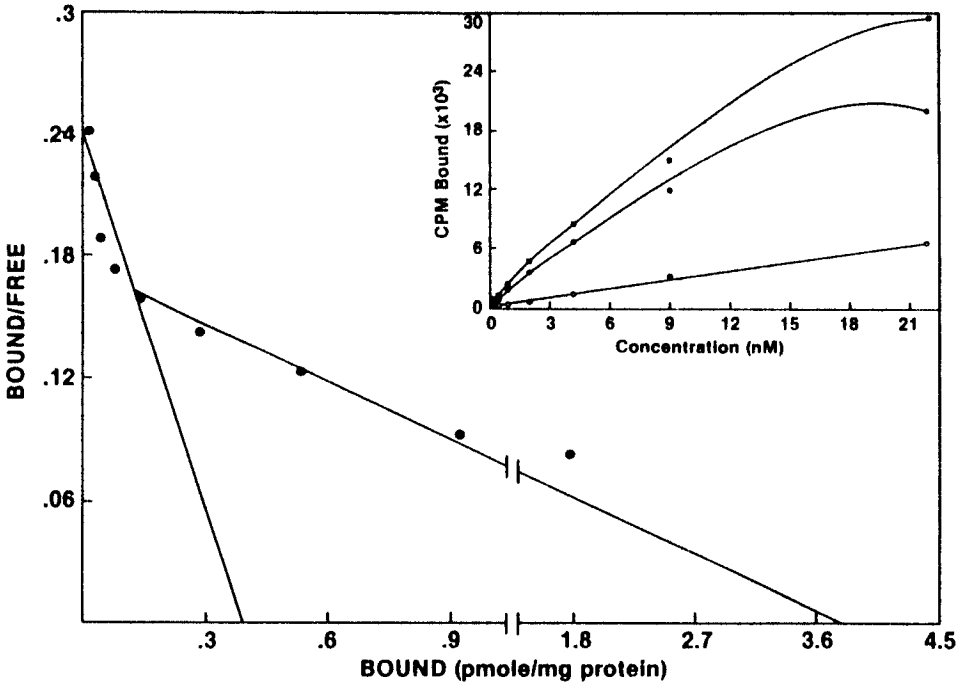

Fig. 1. A typical Scatchard plot of specific (^{3}H)-dexoxadrol binding to rat brain membranes. For every experiment, 8–9 concentrations of (^{3}H) dexoxadrol, each in triplicate, were used. Each point is a mean of seven separate experiments. Each line was fitted by linear regression analysis. Inset: saturation isotherm, total binding, ■; specific binding, ●; nonspecific binding, ○.

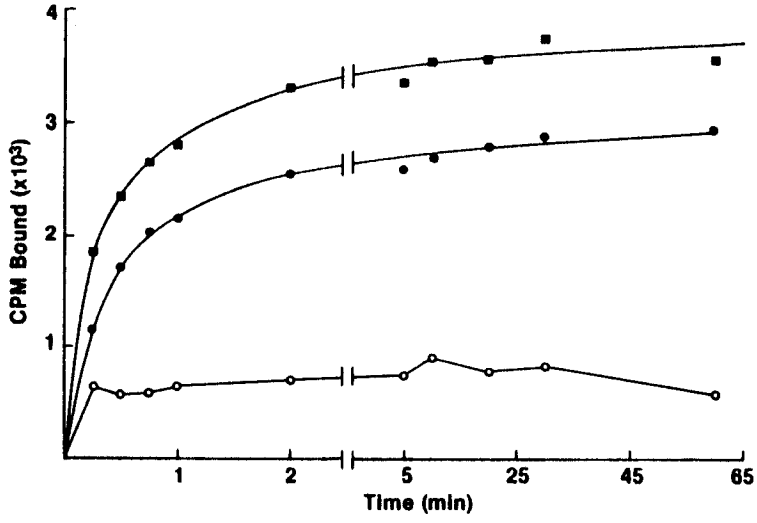

Fig. 2. High-affinity binding of (^{3}H)-dexoxadrol (2 nM) as a function of time. Total binding, ■; specific binding, ●; nonspecific binding, ○.

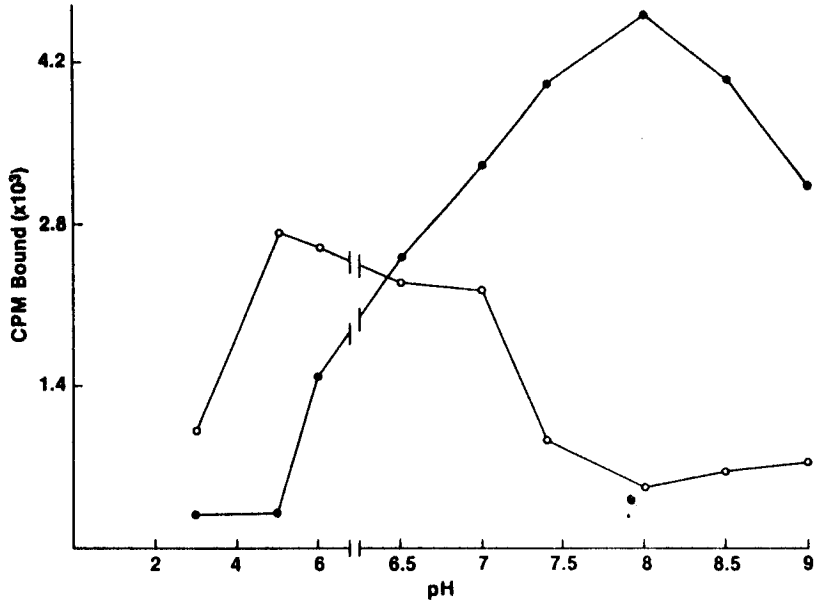

Fig. 3. High-affinity binding of (^{3}H)-dexoxadrol (2 nM) as a function of pH. Specific binding, ●; nonspecific binding, ○.

(^{3}H)-Dexoxadrol Binding as a Function of Protein Concentration

The effect of protein concentration on specific binding of (^{3}H)-dexoxadrol was studied at a concentration range of 0.095 to 1.26 mg protein/ml. The specific binding increased linearly up to about 0.36 mg protein/ml. At higher concentrations, the increase in specific binding was smaller as compared to that being observed at low concentrations (Fig. 4).

Regional Distribution of (^{3}H)-Dexoxadrol Binding in Rat Brain

The specific binding of (^{3}H)-dexoxadrol was highest in the striatum and cerebral cortex. Binding was lowest in the cerebellum. Intermediate levels of binding were observed in the hippocampus, thalamus plus hypothalamus, and brainstem (Table 1).

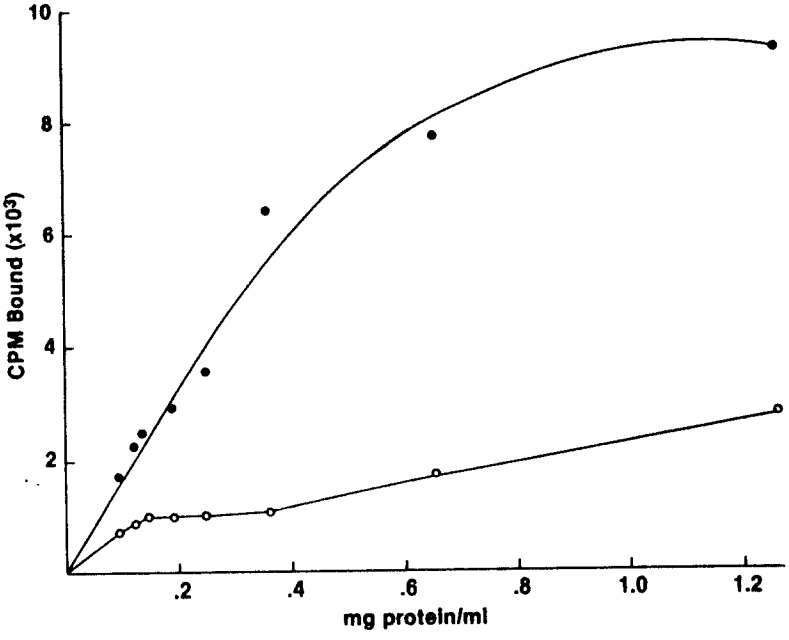

Fig. 4. High-affinity binding of (^{3}H)-dexoxadrol (2 nM) as a function of protein concentration. Specific binding, ●; nonspecific binding, ○.

TABLE 1. Regional Distribution of High-Affinity (^{3}H)-Dexoxadrol (1.99 nM) Binding in Rat Brain

Region	Specific (^{3}H)-dexoxadrol binding: fmol/mg protein[a]
Striatum	313 ± 11.4
Cerebral cortex	308 ± 20.0
Hippocampus	251 ± 6.4
Thalamus + hypothalamus	162 ± 18.9
Brainstem	88 ± 11.9
Cerebellum	76 ± 9.4

[a]Each observation is the mean ± S.E. of four independent experiments.

Effect of Proteolytic Enzymes, Denaturation of Protein and Sulfhydryl Group Inhibitor on (^{3}H)-Dexoxadrol Binding

Preincubation of the membrane suspension with trypsin decreased specific binding of (^{3}H)-dexoxadrol. This decrease was dependent on the concentration of trypsin. Under similar conditions, there was no effect on nonspecific binding. Similar effects were observed with chymopapain, proteinase K, and thermolysin. Denaturation of protein by boiling the membrane suspension for 30 minutes reduced binding of (^{3}H)-dexoxadrol to 33% of control (Table 2).

p-Chloromercuriphenyl sulfonic acid at 1 mM concentration decreased specific binding of (^{3}H) dexoxadrol to 40% of control. 2-Mercaptoethanol at a 1 mM concentration had no significance on binding. However, preincubation of the membrane suspension with 1 mM 2-mercaptoethanol significantly blocked PCMPSA-induced reduction in binding (Table 3).

Competitive Binding Assays and Displacement of Bound (^{3}H)-Dexoxadrol

Among the drugs investigated in competitive binding assay, dexoxadrol was found to be most potent in inhibiting the binding of (^{3}H)-dexoxadrol. The inhibition constants of dexoxadrol and its close analogs were as follows (Ki in nM): dexoxadrol, 8.44; levoxadrol, 48.38; etoxadrol, 68.76; phencyclidine, 670; (+)PCMP, 201.9; and (−)PCMP, 913.8. Among the psychotomimetic opiate derivates, (−)cyclazocine was about twice as potent as (+)cyclazocine, and (−)SKF 10047 (N-allylnormetazocine) was 1.6 times more potent than its (+) isomer in inhibiting (^{3}H)-dexoxadrol binding. Both (+) and (−)amphetamine, diazepam, LSD, mescaline, nifedipine, verapamil, nicotine, DMPP, tetraethylammoniumchloride, and d-tubocurarine were inactive (Table 4). In an investigation to study displacement of bound (^{3}H)

TABLE 2. Effect of Proteolytic Enzymes and Denaturation of Protein on High-Affinity (^{3}H)-Dexoxadrol (2 nM) Binding to Whole Rat Brain Membrane Preparation

Name	Concentration (μg/ml)	Specific binding (% of control)
Trypsin	0	100
	1	62
	3	59
	10	51
	30	38
	100	21
Chymopapain	0	100
	3	67
	10	64
	30	55
Proteinase K	0	100
	0.3	71
	1	65
	3	62
	10	53
	30	31
Thermolysin	0	100
	10	70
	30	68
	100	51
Boiling (20 min)	—	33

TABLE 3. Effect of Sulfhydryl Group Inhibitor on High-Affinity (^{3}H)-Dexoxadrol (2 nM) Binding to Whole Rat Brain Membrane Preparation

Treatment	Concentration (mM)	Specific binding (% of control)
Control	—	100
p-Chloromercuriphenyl sulfonic acid (PCMPSA)	1	40
2-mercaptoethanol	1	94
2-mercaptoethanol + PCMPSA	1,1	85

TABLE 4. Inhibition of High-Affinity (^{3}H)-Dexoxadrol Binding by Various Drugs in Rat Brain Membrane Preparation

Drug	Ki (nM)[a]
Dexoxadrol	8.4
Levoxadrol	48.3
Etoxadrol	68.7
Phencyclidine	670.0
(+) PCMP	201.9
(−) PCMP	913.8
(±) Cyclazocine	539.3
(+) Cyclazocine	534.3
(−) Cyclazocine	281.8
(±) SKF 10,047	1,059.0
(+) SKF 10,047	1,408.9
(−) SKF 10,047	866.3
Methylphenidate	1,138
(+)-Amphetamine	10,000
(−)-Amphetamine	10,000
Benztropine	1,271.3
Diazepam	10,000
LSD	10,000
Mescaline	10,000
Nifedipine	10,000
Verapamil	10,000
Nicotine	10,000
DMPP	10,000
Tetraethylammonium	10,000
d-tubocurarine	10,000

[a]Concentration of (^{3}H)-dexoxadrol, 1.98 nM; Kd_1, 1.68 nM.

dexoxadrol, aliquots of membrane suspension were allowed to reach equilibrium with (^{3}H)-dexoxadrol for 30 minutes at 25°C. Then PCP was added. Within 1 minute after the addition of PCP, specific binding was reduced to 48% of control. This suggested that the t½ is about one minute. All the bound (^{3}H)-dexoxadrol was displaced 60 minutes after the addition of PCP. There was no significant change in nonspecific binding during this period of the investigation (Fig. 5).

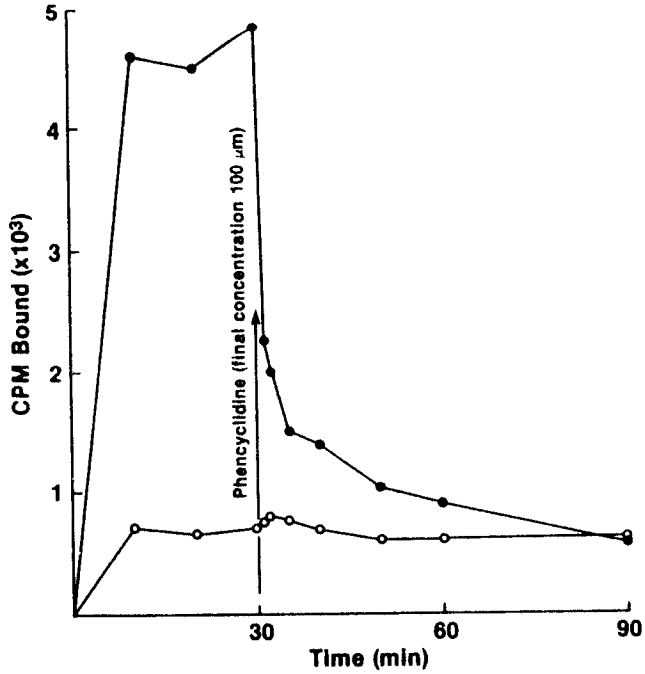

Fig. 5. Displacement of bound (^{3}H)-dexoxadrol (1.98 nM) at various time intervals after the addition of 100 μM PCP. Specific binding, ●; nonspecific binding, ○.

DISCUSSION

The binding isotherm of (^{3}H)-dexoxadrol to whole rat brain demonstrated high- and low-affinity binding sites, with dissociation constants of 1.68 and 26 nM, respectively. The corresponding Bmax values were 0.4 and 3.7 pmol/mg protein. The Bmax of the low-affinity binding site of (^{3}H)-dexoxadrol is close to the reported Bmax of (^{3}H)-PCP binding, 2 to 3.5 pmol/mg protein [Zukin and Zukin, 1979; Zukin et al., 1983; Vincent et al., 1979]. PCP has a low-binding affinity. Because of this property of PCP, it is likely that previous investigators [Zukin et al., 1983; Vincent et al., 1979] may have missed the high-affinity binding site, although Eldefrawi et al. [1982] and Quirion et al [1981a] did demonstrate a PCP binding site with a Kd of 13.5 (crayfish muscle membrane) and 46 nM (slices of rat olfactory bulb), respectively. These Kd values of PCP binding are significantly lower than those reported by earlier investigators [0.15 to 0.2 μM, Zukin and Zukin, 1979; Vincent et al., 1979], suggesting the possibility of a high affinity PCP binding site. With the use of (^{3}H)-dexoxadrol in the binding asays, we have clearly demonstrated both the high- and low-affinity binding sites.

In earlier work, a (^{3}H)-PCP binding assay did not work in our laboratory. Thus we cannot prove that (^{3}H)-dexoxadrol labels the same binding sites to which PCP binds. Since dexoxadrol is a biolog of PCP and the Bmax of the low-affinity binding site of (^{3}H)-dexoxadrol is similar to the Bmax of PCP binding, we can assume that these two ligands may be labeling the same low-affinity binding site.

In regional distribution studies with (^{3}H)-dexoxadrol, the highest number of binding sites were located in the striatum and cerebral cortex, and the lowest were in the cerebellum. This rank order of distribution of the high-affinity binding site in the tissues investigated is in agreement with the observations of Vincent et al. [1979], who investigated regional distribution of binding sites in rat brain at a 1 nM concentration of (^{3}H)-PCP.

Experimental evidence indicates that the high-affinity (^{3}H)-dexoxadrol binding site is a protein. (^{3}H)-Dexoxadrol binding increased with increasing concentrations of protein. Proteolytic enzymes like trypsin, chymopapain, proteinase K, and thermolysin significantly decreased (^{3}H)-dexoxadrol binding, and this effect was dependent upon the concentration of enzymes. The specific binding of (^{3}H)-dexoxadrol was also significantly reduced by denaturation of protein. Preincubation of membrane suspension with 1 mM PCMPSA significantly lowered (^{3}H)-dexoxadrol binding, and this effect could be blocked by prior treatment with 1 mM 2-mercaptoethanol. These observations suggest the presence of a sulfhydryl group in or near the proteinous (^{3}H)-dexoxadrol binding site. Similar characteristics have been reported with PCP binding site at 1 nM [Vincent et al., 1982] and 10 nM [Zukin et al., 1983] concentrations of (^{3}H)-PCP.

Phencyclidine is a basic drug whose pKa appears to be approximately 8–9. It is protonated at acidic pH. Acidification of urine increases renal excretion of PCP in humans because protonated PCP is not reabsorbed in renal tubules [Domino and Wilson, 1977; Perez-Reyes et al., 1982]. The percentage of unbound PCP increases with decreasing pH in both dog and human serum [Owens et al., 1983]. In the (^{3}H)-dexoxadrol binding assay, a pH below 7.4 significantly enhanced nonspecific binding indicating that PCP, in its protonated form, is no longer able to occupy some of the binding sites that it did at pH 7.4 and above. These binding sites are now occupied with (^{3}H)-dexoxadrol, leading to an increase in nonspecific binding.

The competitive binding experiments suggested that (^{3}H)-dexoxadrol binding was inhibited by drugs which are pharmacologically similar to PCP. The order of potency was: dexoxadrol > levoxadrol > etoxadrol > phencyclidine. (+)PCMP was more potent than (−)PCMP in inhibiting (^{3}H)-dexoxadrol binding. This is consistent with the observations of Quirion et al [1981b] with a PCP binding assay. (−) Isomers of both cyclazocine and SKF 10047 were more potent than (+) isomers in inhibiting (^{3}H)-dexoxadrol. Similar stereospecific effects with cyclazocine and SKF 10047 have been reported with (^{3}H)-PCP binding [Zukin, 1982]. (−)Cyclazocine was also reported to be more like PCP than (+)cyclazocine in PCP-like discriminative behavior [Shanon, 1982]. The competitive binding studies that were carried out with (^{3}H)-dexoxadrol agree with (^{3}H)-PCP (concentration: 8–10 nM) binding with respect to stereospecificity, further suggesting that these two ligands may bind to the identical site.

Bound (^{3}H)-dexoxadrol was rapidly displaced by the addition of PCP, confirming an existence of a biological binding site. The displacement was rapid ($t_{1/2}$ = about 1 minute) and was completed within 60 minutes.

Thus, the results with (^{3}H)-dexoxadrol binding indicate that 1) there are high- and low-affinity binding sites, 2) (^{3}H)-dexoxadrol and (^{3}H)-PCP may bind to identical binding sites, 3) (^{3}H)-dexoxadrol has a very low nonspecific binding to glass fiber filter paper, and 4) (^{3}H)-dexoxadrol binding assay could be used for screening PCP analogs.

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