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Demonstration of [^{3}H]Cyclazocine Binding to Mutiple Opiate Receptor Sites

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SUMMARY

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Interaction of the psychotomimetic opiate cyclazocine with multiple receptor sites has been demonstrated biochemically. Cyclazocine has been postulated to interact with μ, κ, and σ opiate receptors (Martin *et al.*, *J. Pharmacol. Exp. Ther.* **197**:517–532 (1976).) In an effort to understand the molecular mechanisms involved in the neuropharmacological actions of this and closely related opiates we have studied the binding of [^{3}H]cyclazocine to rat brain homogenates. Specific binding, defined as total binding minus binding in the presence of 10 μM nonradioactive cyclazocine, constitutes approximately 92% of total binding at 1.0 nM ^{3}H-labeled ligand and 67% of total binding at 100 nM ^{3}H-labeled ligand. Scatchard analyses utilizing various competing drugs reveal the apparent interaction of this drug with three distinct binding sites characterized by affinities of 0.2 nM, 11 nM, and 70 nM (50 nM Tris-HCl buffer, pH 7.4 at 4°). In contrast, many radiolabeled classical opiates and opioid peptides have been reported to exhibit biphasic binding but do not exhibit binding to a site of such low affinity. The high- and low-affinity [^{3}H]cyclazocine sites exhibit differential sensitivities to sodium and also to the selective sulfhydryl reagent *N*-ethylmaleimide. In addition, all three sites exhibit greater than 50% loss of specific binding following incubation with trypsin (5 μg/ml) for 15 min at room temperature, and greater than 80% loss of specific binding following incubation at 60° for 15 min in the absence of added reagents. Together, these findings indicate that all three sites have a protein-like component. Competition analyses involving rank order determinations for a series of opiates and other drugs indicate that the cyclazocine binding sites represent, in order of decreasing affinity, the classical opiate receptor (the putative "μ" receptor), a second as yet uncharacterized opiate binding site, and the specific [^{3}H]phencylidine binding site. Specific [^{3}H]phencyclidine binding can be displaced by cyclazocine (IC_{50} = 350 nM) and by related benzomorphans, but not by classical opiates such as morphine or naloxone. We thus propose a common binding site in rat nervous tissue for phencyclidine and some of the benzomorphan opiates.

INTRODUCTION

On the basis of behavioral and neurophysiological evidence, Martin and co-workers (1) postulated the existence of heterogeneous opiate receptors. Striking differences in pharmacological responses to different types of narcotics and in their abilities to substitute for one another in the suppression of withdrawal symptoms in addicted animals led these investigators to propose the existence of at least three receptor types in the central nervous system of dogs. These were (a) μ receptors with which morpine and other classical opiates preferentially interact, (b) κ receptors for which the prototypic ligand is the benzomorphan ketocyclazocine, and (c) σ receptors with which some benzomorphans such as cyclazocine and SKF 10,047[2] interact.

Lord *et al.* (2) and subsequently others (3–8) demonstrated pharmacological and biochemical evidence for the existence of heterogeneous opiate receptor populations in several different tissues. In particular, studies

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[2] The abbreviations used are: SKF 10,047, *N*-allylnorcyclazocine; PCP, phencyclidine [N-(1-phenylcyclohexyl) piperidine]; FK 33-824, D-Ala^2-*N*-Me-Phe^4,Met-(*O*)^5-ol-enkephalin; NEM, *N*-ethylmaleimide; DTT, dithiothreitol.

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involving competition of ligands for radiolabeled opiate binding in brain (2–6) and cross-protection studies involving inactivation of opiate binding by phenoxybenzamine and selective sulfhydryl reagents (7, 8) have provided biochemical evidence for μ and the putative δ receptors (with which the shorter enkephalin peptides preferentially interact) and indicated that these have somewhat different distributions throughout the brain. However, no direct biochemical evidence for the existence of distinct κ or σ receptors has yet been obtained.

Cyclazocine is an opiate of the benzomorphan group that has been postulated to interact with μ, κ, and σ receptors (1). Cyclazocine differs from classical opiates in displaying psychotomimetic effects in humans and unique behavioral effects in animals (9, 10). The complex actions of this drug vary with the dose administered. At low doses, cyclazocine produces both morphine agonist actions such as analgesia (11) and antagonist actions such as precipitation of withdrawal in morphine-addicted subjects (12). At high doses, cyclazocine produces a combination of sedation, "drunkenness," and psychosis differing from any morphine effect (13, 14). In an effort to understand the molecular mechanisms involved in the neuropharmacological actions of this and closely related opiates, we have studied the binding of [^{3}H]cyclazocine to rat brain homogenates. In a direct study of [^{3}H]cyclazocine binding to rat nervous tissue, we here demonstrate the interaction of this drug with three binding sites. We propose that these may represent (in order of decreasing cyclazocine-binding affinity) the classical opiate, or "μ" receptor; an intermediate site; and the "σ" receptor, which is here identified with the specific [^{3}H]PCP binding site (15, 16).

MATERIALS AND METHODS

Cyclazocine and SKF-10,047 were gifts from Dr. Arthur Jacobson, National Institutes of Health, Bethesda, Md.; nalorphine and pentazocine, from Dr. Eric Simon, New York University School of Medicine, New York, N. Y.; metazocine, from Dr. Agu Pert, National Institute of Mental Health, Bethesda, Md.; and ethylketocyclazocine, ketocyclazocine, and levallorphan, from Dr. Alan Gintzler, Columbia University College of Physicians and Surgeons, New York, N. Y.; Levorphanol was generously provided by Hoffmann-La Roche Inc., Nutley, N. J.; ketamine, by Bristol Laboratories, Syracuse, N. Y.; PCP and its analogs, by the National Institute on Drug Abuse, Bethesda, Md.; and cyclorthan, etazocine, and WIN derivatives by Sterling-Winthrop, Inc., New York, N. Y. FK 33-824 and other opioid peptides were obtained from Peninsula Laboratories, Inc., San Carlos, Calif. NEM was purchased from Aldrich Chemical Company; Milwaukee, Wisc., DTT from Calbiochem, San Diego, Calif. [^{3}H]Cyclazocine (4.7 Ci/mmole) was generously provided by the National Institute on Drug Abuse; [Piperidyl 3,4-^{3}H]PCP (48 Ci/mole) was obtained from New England Nuclear Corporation, Boston, Mass.

Male Sprague-Dawley rats (150–200 g) were rapidly killed by decapitation, and the whole brains or regions were removed and used to prepare homogenate as previously described (15–18). The weights of brain regions (tissues pooled from two animals) were as follows: cerebellum, 406 mg; frontal cortex, 88 mg; hypothalamus, 214 mg; hippocampus, 160 mg; striatum, 88 mg; brain stem, 40 mg.

[^{3}H]PCP filter binding assays were carried out as previously described (15) and [^{3}H]cyclazocine assays by a modification of this procedure. Aliquots of freshly prepared homogenate (2.0 ml, 1.5 mg of protein) or, in the case of regional studies (1.0 ml, 0.3 mg of protein), in 50 mM Tris-HCl buffer (pH 7.4) were incubated in triplicate at 4° for 45 min with various concentrations of [^{3}H]cyclazocine in the absence or presence of 10 μM nonradioactive cyclazocine and/or other drugs as indicated. In the case of the filter assay, free ligand was separated from membrane-bound [^{3}H]cyclazocine by filtration under reduced pressure through GF/B glass fiber filters (Whatman) that had been presoaked in 0.01% polylysine for 2 hr at 4°. The filters were rapidly washed with two aliquots of 10 ml of 50 mM Tris-HCl (pH 7.4, 4°). Filters were then transferred to Aquasol/toluene (2:1, v/v) and assayed by liquid scintillation spectrometry (Intertechnique ABAL SL 40) at a counting efficiency of approximately 50%. Presoaking the filters decreased the radioactivity adsorbed to them from approximately 20% to less than 1% of specifically bound ligand found when whole brain homogenates (0.75 mg/ml of protein) were included in the assay. Specific [^{3}H]cyclazocine binding is defined as total binding minus the binding in the presence of 10 μM cyclazocine. Membrane protein concentration was determined by the method of Lowry *et al.* (17).

In the case of the centrifugation assay, tissue preparation and incubations were carried out in triplicate as described above. Free ligand was separated from membrane-bound [^{3}H]cyclazocine by centrifugation of 0.5-ml samples in 1.5-ml microfuge tubes at 30,000 × *g* for 20 min. The supernatant was discarded, the pellet was rinsed once with 1.0 ml of ice-cold buffer, and the microfuge tube was wiped dry. The end of the tube containing the pellet was then severed with a scalpel blade and counted as above.

For inactivation studies, aliquots of homogenate (2 ml, 1.5 mg of protein) were incubated in 50 mM Tris-HCl (pH 7.4, 37°) in the presence or absence of NEM (0.5 mM) for times as indicated (18). The reaction was stopped by addition of excess DTT (2.5 mM); samples were then incubated for an additional 30 min at 4° with [^{3}H]cyclazocine (0.2–200 nM) in the absence or presence of nonradioactive cyclazocine (10 μM) and filtered as above.

RESULTS

Equilibrium binding of [^{3}H]cyclazocine to rat brain homogenates. Specific [^{3}H]cyclazocine binding, defined as the total binding minus the binding in the presence of 10 μM cyclazocine (determined by the rapid filtration method), was saturable with respect to radiolabeled ligand concentration (Fig. 1A). Specific binding constituted approximately 92% of total binding (at 1.0 nM ^{3}H-labeled ligand) and 67% of total binding (at 100 nM ^{3}H-labeled ligand). In separate control experiments employing the centrifugation assay (Fig. 1C), similar results were obtained. Half-maximal binding in both experiments oc-

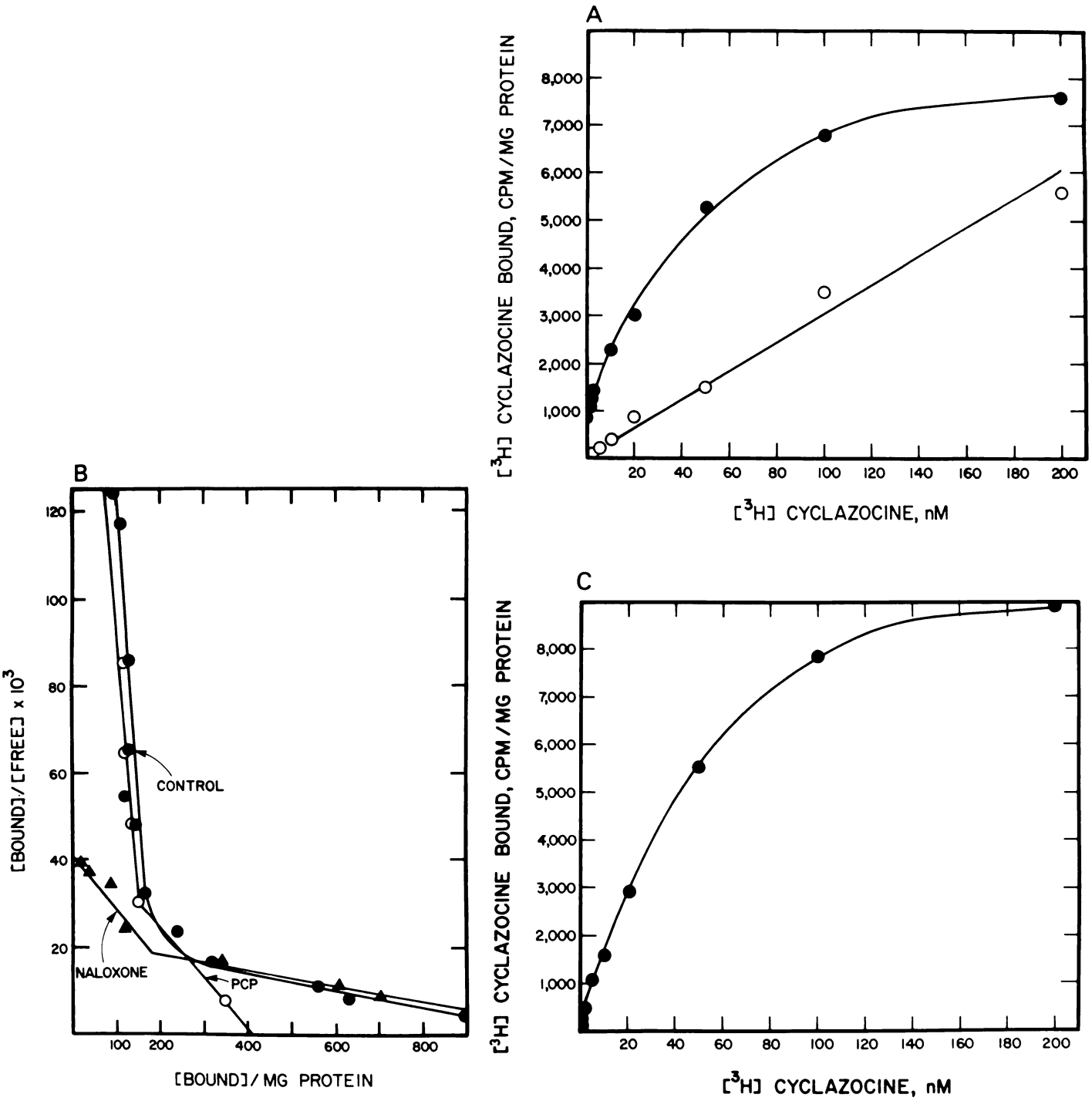

FIG. 1. *Specific binding of [^{3}H]cyclazocine to rat brain homogenates (A) as a function of ligand concentration (● specific binding, ○ nonspecific binding); (B) as a Scatchard analysis (● in the absence of other added drugs, ▲ in the presence of 15 nM naloxone, ○ in the presence of 10 μM PCP); (C) specific [^{3}H]cyclazocine in the centrifugation assay*

Aliquots of homogenate (2 ml, 1.5 mg of protein) in 50 mM Tris-Cl (pH 7.4) were incubated in triplicate at 4° for 45 min with various concentrations of [^{3}H]cyclazocine and other indicated drugs in the absence or presence of 10 μM nonradioactive cyclazocine. Free [^{3}H]cyclazocine was separated from bound ligand by the rapid filtration method. Specific binding, defined as total binding minus binding in the presence of nonradioactive cyclazocine, is reported. Data are the means from three independent experiments, each carried out in triplicate. Data as shown were fit by straight lines; the biphasic plot for the control case was subsequently analyzed using a computer program for nonlinear least-squares regression analysis as described in the text. In C, specific [^{3}H]cyclazocine binding to rat brain tissue was determined with the centrifugation assay. Samples were incubated as above; free [^{3}H]cyclazocine was separated from bound ligand by centrifugation as described under Materials and Methods.

TABLE 1

Relative potencies of drugs in reducing low-affinity [^{3}H]cyclazocine binding or [^{3}H]PCP binding to rat brain homogenates

Whole rat brain was used to prepare homogenate in 50 mM Tris-HCl buffer (pH 7.4) as described under Materials and Methods. In binding analyses using [^{3}H]cyclazocine, aliquots of freshly prepared homogenate (1.0 ml, 0.75 mg pf protein) were incubated in triplicate at 4° for 45 min with 70 nM [^{3}H]cyclazocine (376,000 cpm) and 70 nM naloxone in the absence or presence of 0.01 mM cyclazocine or other indicated drugs. Control binding was 1650 cpm specifically bound per sample. In analyses using [^{3}H]PCP, samples were incubated under the same conditions with 7.0 nM [^{3}H]PCP (New England Nuclear Corporation, 48 Ci/mmole, 420,000 cpm/sample), alone or in the presence of 0.1 mM PCP or other indicated drugs. Control binding was 3200 cpm specifically bound per sample. Samples were filtered and assayed for radioactivity as described under Materials and Methods.

Drug[a]	Displacement of IC_{50}[b]	Relative potency of 70 nM [^{3}H]cyclazocine[c]	Displacement of IC_{50}[b]	Relative potency of [^{3}H]PCP
	μM		μM	
Benzomorphan opiates				
Cyclazocine	0.38 ± 0.01	1.0	0.35 ± 0.03	1.0
SKF 10,047	0.97 ± 0.08	0.40	1.0 ± 0.06	0.35
Cyclorphan	0.98 ± 0.05	0.39	1.6 ± 0.05	0.23
Etazocine	2.5 ± 0.08	0.15	4.5 ± 0.05	0.08
Ketocyclazocine	2.4 ± 0.1	0.16	9 ± 0.25	0.04
Ethylketocyclazocine	3.8 ± 0.1	0.10	13 ± 0.30	0.03
Pentazocine	10 ± 0.8	0.038	12 ± 2.2	0.02
Nalorphine	15 ± 1.2	0.025	>100 ± 5.0	0.002
Levallorphan	20 ± 1.5	0.019	75 ± 5.0	0.003
Dihydromorphine	21 ± 2.0	0.018	>50	>0.007
Naloxone	>50	<0.008	>50	>0.007
D-Ala^2,D-Leu^5-enkephalin	>50	<0.008	>50	>0.007
Phencyclidine and its derivatives				
PCP	0.6 ± 0.05	1.0	0.23 ± 0.01	1.0
1-[1-(2-Thienyl)-cyclohexyl]-piperidine	0.45 ± 0.04	1.33	0.16 ± 0.01	1.44
N-Ethyl-1-phenylcyclohexylamine	0.55 ± 0.04	1.2	0.14 ± 0.02	1.6
1-(1-Phenylcyclohexyl)-pyrrolidine	0.6 ± 0.05	1.0	0.2 ± 0.01	1.2
1-[1-(2-Thienyl)-cyclohexyl]-pyrrolidine	0.85 ± 0.05	0.7	0.3 ± 0.02	0.8
N,N-Dimethyl-1-phenyl-cyclohexylamine	2.0 ± 0.09	0.30	0.5 ± 0.02	0.45
1-[1-(2-Thienyl)-cyclohexyl]-morpholine	3.0 ± 1.0	0.20	1.6 ± 0.09	0.14
1-(1-Phenylcyclohexyl)-morpholine	5.5 ± 1.0	0.11	2.4 ± 0.04	0.10
1-Piperidinocyclohexane-carbonitrile	50 ± 5.0	0.02	5.5 ± 0.20	0.04

[a] Drugs which proved inactive at displacing low-affinity [^{3}H]cyclazocine binding or [^{3}H]PCP binding at 100 μM: naloxone, morphine, and the opioid peptides D-Ala^2,Met^5-enkephalin, D-Ala^2,Leu^5-enkephalin, and D-Ala^2-β-endorphin.

[b] IC_{50}, Concentration displacing 50% of bound [^{3}H]PCP or of bound [^{3}H]cyclazocine. Results are the means ± standard error of the mean from a minimum of three experiments, each carried out in triplicate.

[c] Data for the benzomorphan opiates are expressed relative to cyclazocine (relative potency = 1). Data for phencyclidine and related drugs are expressed relative to phencyclidine (relative potency = 1).

curred at approximately 30 nM [^{3}H]cyclazocine. In contrast, nonspecific binding, indicated by the binding of [^{3}H]cyclazocine in the presence of 10 μM cyclazocine, was not saturable and increased linearly with increasing ^{3}H-labeled ligand. Stereospecific binding, defined as binding in the presence of 1 μM dextrorphan minus binding in the presence of 1 μM levorphanol (determined by the rapid filtration assay), varied less than ±5% from specific binding over the concentration range 0.5 nM–50 nM [^{3}H]cyclazocine.

When the binding data shown in Fig. 1A were replotted in a Scatchard analysis (Fig. 1B), a biphasic curve was observed which can be interpreted as revealing at least two binding sites. When these data were analyzed using a computer program for nonlinear weighted, least-squares regression analysis,[3] a good fit was obtained for a curve calculated for two binding components. The first had a K_d of 0.18 ± 0.1 nM and B_{max} = 103 ± 0.01 fmoles/mg of protein; the second, a K_d of 70 ± 13 nM, and B_{max} = 1.0 ± 0.08 pmoles/mg of protein. Because the affinities differed approximately 350-fold, graphical analysis yielded relatively similar results.

Specific binding of [^{3}H]cyclazocine (1 nM or 70 nM) to rat brain homogenates was found to increase linearly between 0.1 and 1 mg/ml of whole rat brain protein. Binding studies were routinely performed within this linear range. Incubation of homogenate with trypsin (1 mg/ml) for 30 min at 30° decreased specific binding of [^{3}H]cyclazocine (1 nM or 70 nM) more than 80% relative to control samples incubated under the same conditions in the absence of trypsin. Incubation with a lower trypsin concentration (5 μg/ml) decreased specific binding approximately 50% at both concentrations of ligand. Finally, incubation of homogenates at 60° for 15 min decreased specific binding of [^{3}H]cyclazocine (1 nM or 70 nM) greater than 80% relative to control samples incubated at 4°.

The apparent K_d of 0.2 nM for the high-affinity cyclazocine binding site is consistent with the ED_{50} of 0.9 nM reported for displacement by cyclazocine of [^{3}H]naloxone

[3] R. E. Reinman and G. W. Pasternak, in preparation.

binding to the opiate receptor (19). Addition of 15 nM naloxone to the incubation mixture (Fig. 1B) resulted in a marked reduction of [^{3}H]cyclazocine binding to the tight sites with relatively little change in binding to the weak sites. When a series of opiates was tested for ability to displace specifically bound [^{3}H]cyclazocine (1 nM) (20), their rank order (etorphine > levallorphan = levorphanol > naloxone > pentazocine) agreed with that for their displacement of [^{3}H]naloxone (19). Together these data indicate that the high-affinity cyclazocine binding is occurring to the classical opiate receptor.

In order to determine the identity of the low-affinity cyclazocine binding site, a variety of drugs, putative transmitters, and receptor ligands was tested for ability to displace higher-concentration [^{3}H]cyclazocine (Table 1). Of the drugs tested, the only drugs other than cyclazocine-like opiates which proved active were PCP-like drugs. The relative potencies of a series of PCP analogues in displacement of [^{3}H]cyclazocine (70 nM) in the presence of naloxone (70 nM) were similar to those for ability to displace [^{3}H]PCP from its binding site (Table 1). The observed rank order (1-[1-(2-thienyl)-cyclohexyl]-piperidine = *N*-ethyl-1-phenylcyclohexylamine > PCP > *N*,*N*-dimethyl-1-phenyl-cyclohexylamine > ketamine > 1-(1-phenylcyclohexyl)-morpholine > 1-[-(2-thienyl)-cyclohexyl]-morpholine > 1-piperidinocycloxhexane-carbonitrile) was similar to that determined for [^{3}H]PCP displacement. The binding affinity of 70 nM observed for [^{3}H]cyclazocine binding to the lower-affinity sites (Fig. 1B) is consistent with the IC_{50} of 350 nM observed for displacement of [^{3}H]PCP (7.0 nM) by cyclazocine (Table 1). The total number of weak cyclazocine binding sites (1000 fmoles/mg of protein) was similar to that of specific PCP sites determined using polylysine-soaked filters (1100 fmoles/mg of protein). Addition of PCP (10 μM) to the incubation mixture (Fig. 1B) resulted in the marked reduction of [^{3}H]cyclazocine binding to the weak (70 nM) sites. Together, these findings suggest that the "weak" [^{3}H]cyclazocine binding is occurring to the [^{3}H]PCP binding site.

Kinetic analysis of high- and low-affinity [^{3}H]cyclazocine sites. In order to determine whether the interaction of [^{3}H]cyclazocine with its binding sites meets the reversibility criterion of receptor-ligand interactions and to determine independently the affinitites associated with each site, kinetic analyses were carried out. The time course of [^{3}H]cyclazocine (2 nM and 80 nM) association to rat brain homogenates was examined (Fig. 2A). At both concentrations, specific drug binding proceeded to a fixed value and was complete within less than 5 min at 4°. The half-times for association of 2 nM and 80 nM ligands were 1.0 min and 0.6 min, respectively. The observed first-order rate constants for association (k_{obs}) were determined (20); these were $k_1 = 9.8 \times 10^7$ M^{-1} min^{-1} for the high-affinity site and $k_1 = 4.6 \times 10^6$ M^{-1} min^{-1} for the low-affinity site. Association constants of $k_1 = 0.4 \times 10^7$ M^{-1} min^{-1} for [^{3}H]dihydromorphine binding at 0° and of $k_1 = 1 \times 10^7$ M^{-1} min^{-1} for [^{3}H]naloxone binding have been reported (21).

Figure 2B shows the time course of the dissociation of specifically bound [^{3}H]cyclazocine (2 nM and 80 nM) from rat brain homogenates. Excess (10 μM) nonlabeled cyclazocine was added to prevent significant rebinding of [^{3}H]-cyclazocine after dissociation. Binding in the case of the high-affinity site declined in a monophasic, exponential manner with $t_{1/2} = 10$ min; binding to the low-affinity site (K_d = 70 nM) declined in a biphasic manner with $t_{1/2} = 3$ min (for the fast component). The half-life for dissociation of [^{3}H]naloxone at 5° is 5 min (22). Dissociation rate constants for cyclazocine of $k_{-1} = 0.069$ min^{-1} and $k_{-1} = 0.23$ min^{-1} were calculated for the tight and weak sites, respectively. Estimates of the binding affinities from the ratios of k_{-1}/k_{+1} for the high- and low-affinity sites were found to be 0.7 nM and 50 nM, respectively.

Differential sensitivities of high- and low-affinity [^{3}H] cyclazocine sites to sodium and NEM. The relative sensitivites of the high- and low-affinity cyclazocine binding sites to both sodium and NEM were examined. Scatchard analyses of [^{3}H]cyclazocine binding to rat brain homogenates in the presence and absence of NaCl (50 mM) were obtained. The presence of sodium resulted in an increase in the affinity associated with the high-affinity sites from 0.2 to 0.1 mM with no apparent change in the number of binding sites (Table 2); no detectable change in either affinity or density of the weak sites was observed. Results similar to those for the high-affinity site have been reported in the case of sodium modulation of classical opiate binding (18, 21). [^{3}H]Cyclazocine binding to homogenates incubated at 37° for 15 min in the presence or absence of NEM (0.5 nM), followed by addition of DTT (2.5 mM) to quench the reaction, was also investigated (Table 2). Scatchard analyses of [^{3}H]cyclazocine binding to NEM-treated homogenates revealed a 40% reduction in the number of high-affinity sites, with no apparent change in affinity relative to control homogenate incubated in the absence of NEM; the number of low-affinity sites was decreased approximately 20% relative to the control, with no apparent change in K_d. NEM was previously found to decrease the apparent number of [^{3}H]dihydromorphine binding sites, with no change in the receptor affinity (22).

The binding of [^{3}H]cyclazocine to rat brain membranes was next examined in the presence of both naloxone (15 nM) and PCP (10 μM) (Fig. 3). In this case both the tight [^{3}H]cyclazocine binding site (K_d = 0.2 nM) and the weak site (K_d = 70 nM) were essentially eliminated; residual binding of [^{3}H]cyclazocine was to approximately 142 fmole sites per milligram of protein with an affinity of 11 nM (r = 0.96). When a series of opiates was tested for ability to displace 11 nM [^{3}H]cyclazocine in the presence of 15 nM naloxone and 10 μM PCP (Table 3), the observed rank order was ethylketocyclazocine > WIN 44,441-3 > ketocyclazocine > D-Ala^2,D-Leu^5-enkephalin > SKF 10,047 > FK 33 824 > naloxone.

Finally, in order to determine the selectivity of the [^{3}H]PCP binding site for opiates, a series of classical opiates, endogenous opioid peptides, and cyclazocine-like opiates was examined for ability to displace [^{3}H]PCP binding *in vitro* (Table 1). The classicial opiates naloxone, etorphine, and morphine, and the opioid peptides D-Ala^2,Met^5-enkephalin, D-Ala^2,Leu^5-enkephalin, and D-Ala^2-β-endorphin at 0.01 mM concentration were unable to displace [^{3}H]PCP. In contrast, series of cyclazocine-like opiates did displace specific [^{3}H]PCP binding. The observed rank order was cyclazocine > SKF 10,047 >

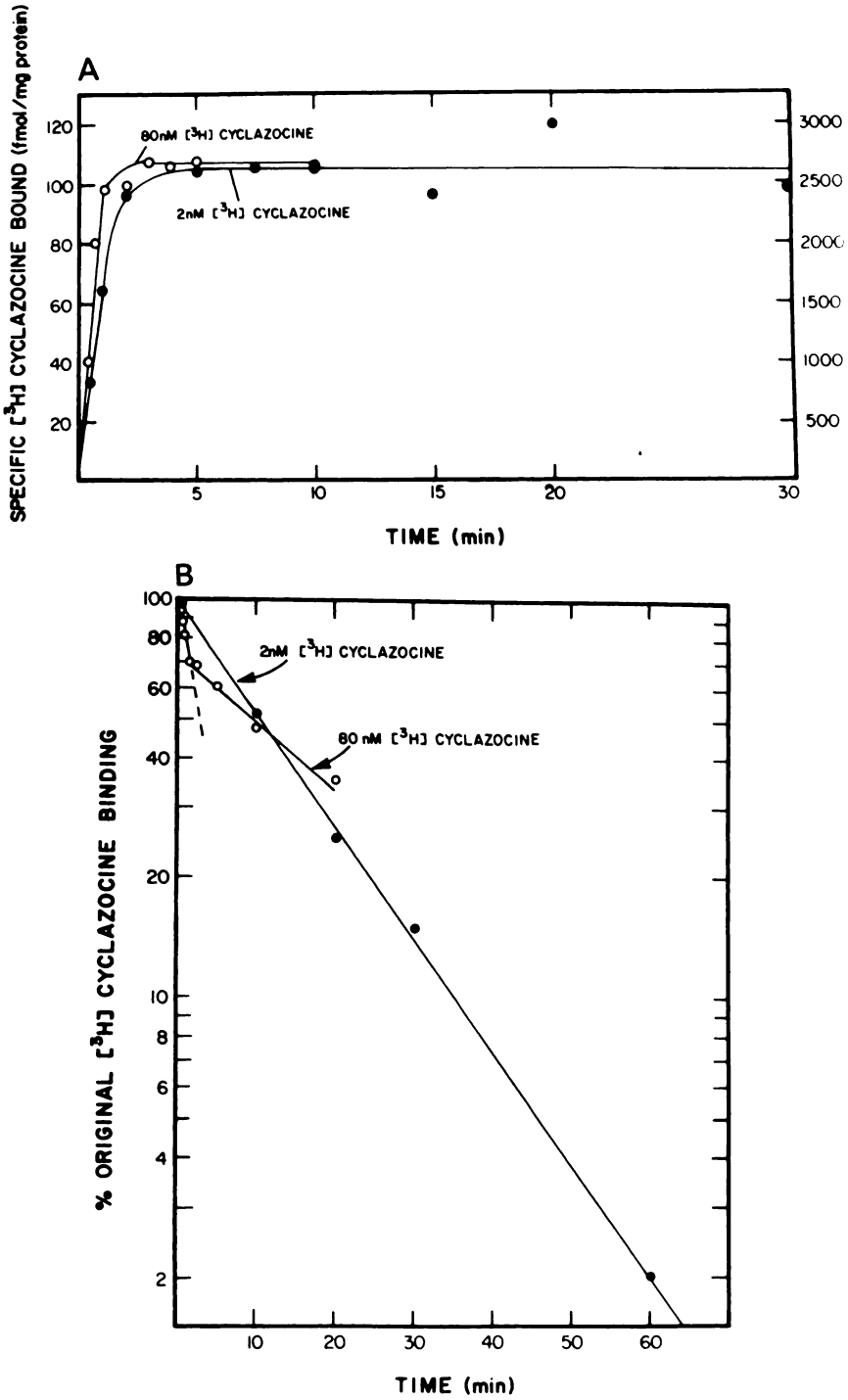

FIG. 2. *Association and dissociation time courses of specifically bound [^{3}H]cyclazocine*

A. Association time course of specifically bound [^{3}H]cyclazocine at two concentrations to rat brain homogenates at 4° [● 2 nM [^{3}H]cyclazocine (*left scale*), ○ 80 nM [^{3}H]M (*right scale*)]. Incubations were carried out as described under Materials and Methods in the presence or absence of 10 μM cyclazocine.

B. Dissociation time course for specifically bound [^{3}H]cyclazocine (● 2 nM [^{3}H]cyclazocine, ○ 80 nM [^{3}H]cyclazocine). Membranes (0.75 mg of protein per milliliter) were incubated with [^{3}H]cyclazocine at the indicated concentration in 50 mM Tris-HCl buffer (pH 7.4) for 45 min at 4°. At time zero, radiolabeled ligand binding was reversed by dilution of the incubation mixture 100-fold into incubation buffer containing 10 μM cyclazocine.

cyclorthan > etazocine > ketocyclazocine > pentazocine > ethylketocyclazocine > levallorphan. Nalorphine proved inactive at 10 μM.

Regional distribution of [^{3}H]cyclazocine binding. In preliminary studies, specific binding of 2 nM [^{3}H]cyclazocine was highest in the caudate nucleus (172 fmoles/mg of protein), cervical spinal cord, and hypothalamus; binding in the cerebellum (12 fmoles/mg of protein) was less than one-tenth that in the caudate nucleus. Intermediate binding was observed in the hippocampus and

TABLE 2

Effect of NEM and sodium on high- and low-affinity cyclazocine binding sites

Binding studies using the rapid filtration assay and inactivation studies were carried out as described under Materials and Methods. Data are the means of three independent experiments, each carried out in triplicate, and have been analyzed using a computer program for nonlinear, weighted, least-squares regression analysis.[3]

Reagent	K_d		n	
	High-affinity sites	Low-affinity sites	High-affinity sites	Low-affinity sites
	nM		*fmoles/mg protein*	
None	0.2 ± 0.1	70 ± 13	103 ± 20	1000 ± 80
NEM (0.5 mM)	0.2 ± 0.1	70 ± 13	62 ± 15	820 ± 80
NaCl (50 mM)	0.07 ± 0.04	70 ± 13	105 ± 20	1000 ± 80

frontal cortex. In contrast, specific binding of 60 nM [^{3}H] cyclazocine in the presence of 15 nM naloxone was highest in the hippocampus (916 fmoles/mg of protein). Binding in the cervical spinal cord (261 fmoles/mg of protein) was only 28% that in the hippocampus. Intermediate binding was found in the hypothalamus, frontal cortex, cerebellum, and striatum. In these studies, binding values in all six regions were compared on the same day in samples adjusted to the same protein concentration (0.3 mg/ml). All results are the average of four independent experiments, each carried out in triplicate.

DISCUSSION

Our data indicate that cyclazocine and related opiates have a complex mode of action which is mediated at multiple binding sites. Of the sites revealed by Scatchard analysis of [^{3}H]cyclazocine binding, that of highest affinity displays biochemical and pharmacological properties characteristic of the classical opiate receptor. Binding at the 11 nM site is displaced most effectively by ketocyclazocine-like opiates. Binding at the lowest affinity site is displaceable by PCP and its derivatives as well as by cyclazocine-like opiates. Thus, this site closely resembles the specific [^{3}H]PCP binding site previously described. Of a large number of opiates tested, only cyclazocine-like drugs were able to bind to the [^{3}H]PCP site. Thus, the present study may provide a molecular model for the complex neuropharmacological actions of cyclazocine-like drugs.

Neurophysiological studies had suggested that cyclazocine interacts at μ, κ, and σ opiate rceptor sites. Inter-

TABLE 3

Relative potencies of drugs in displacing 11 nM [^{3}H] cyclazocine binding to rat brain homogenates

Results are the means ± standard error of the mean from three experiments, each carried out in triplicate. Whole rat brain was used to prepare homogenate in 50 mM Tris-HCl buffer (pH 7.4) as described under Materials and Methods. Aliquots of homogenate (2.0 ml, 1.5 mg of protein) were incubated in triplicate at 4° for 45 min with 11 nM [^{3}H] cyclazocine, 15 nM naloxone, and 10 μM PCP in the absence or presence of 10 μM cyclazocine or other indicated drugs. Samples were filtered and assayed for radioactivity as described for Fig. 1. Specific binding in controls was approximately 1220 cpm bound per sample.

Drug	IC_{50}	Relative potency
	nM	
Cyclazocine	10 ± 1.0	1.0
Ethyl ketocyclazocine	18 ± 1.8	0.88
WIN 44,441-3	28 ± 5.0	0.36
Ketocyclazocine	50 ± 5.0	0.20
D-Ala^2,Met^5-enkephalin	60 ± 5.0	0.16
Levorphanol	62 ± 5.0	0.16
SKF 10,047	100 ± 10.0	0.10
FK 33-824 enkephalin	105 ± 10.0	0.09
Morphine	210 ± 10.0	0.05
Naloxone	50	0.20
1-[1-(2-Thienyl)-cyclohexyl]-piperidine	>1000	<0.01

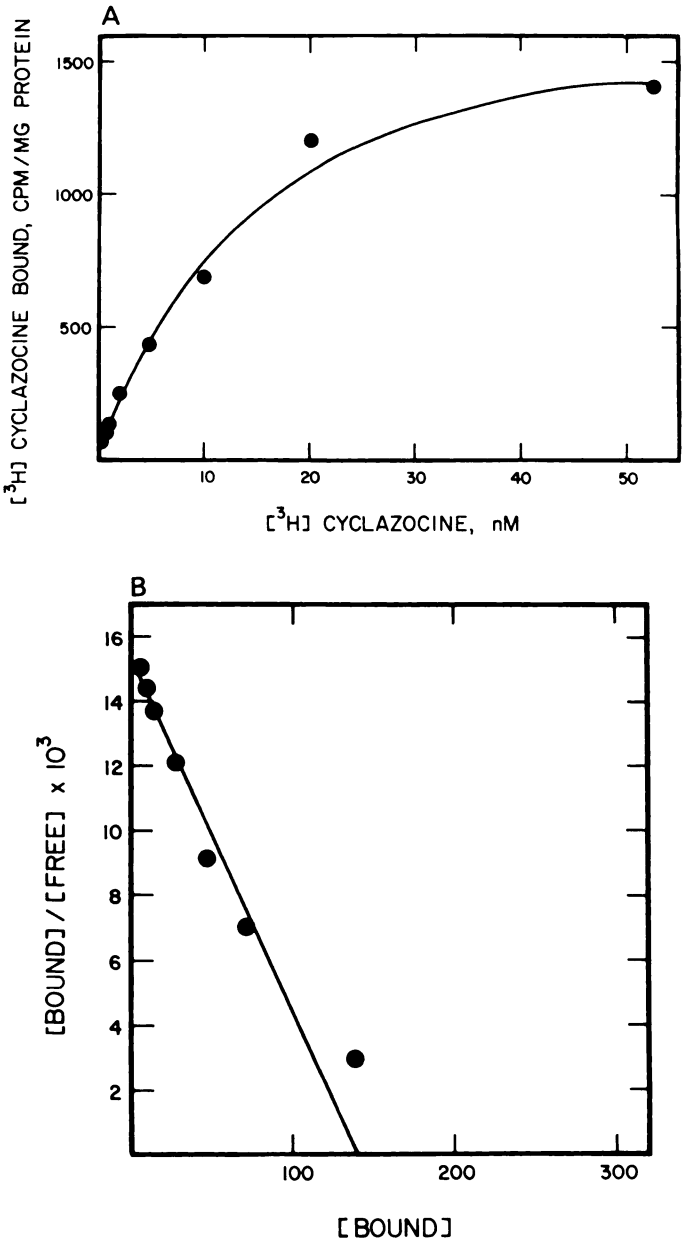

FIG. 3. *Specific binding of [^{3}H]cyclazocine to rat brain homogenates in the presence of 15 nM naloxone and 10 μM PCP (A) as a function of ligand concentration and (B) as a Scatchard analysis*

Binding was carried out as described in the legend to Fig. 1. Data are the means of three independent experiments, each carried out in triplicate. Specific binding was 50% of total binding at 50 nM [^{3}H] cyclazocine. Data were fit by a straight line using linear regression analysis.

actions at the σ sites were thought to produce the "canine delirium" as well as tachycardia and tacypnea in dogs and the "hallucinogenic and aversive" effects of the psychotomimetic opiates in man (1). Our findings provide biochemical substantiation for the interaction of cyclazocine at the morphine (μ) or classicial opiate site (based on biochemical and pharmacological properties of the high-affinity site) and at a second (70 nM) site which may be the σ site.

Several pieces of evidence suggest that the σ receptor and PCP binding site may be the same. Thus, of a large number of opiates tested, only cyclazocine-like opiates have been shown to displace [^{3}H]PCP binding; conversely, PCP and its derivatives can inhibit binding of [^{3}H]cyclazocine to its lowest affinity (K_d = 70 nM) binding site. Behaviorally, too, the two classes of drugs can produce similar effects. Maayani and Weinstein (23) had suggested that the filtration method might be unsuitable for detecting the pharmacologically relevant binding sites of [^{3}H]PCP. More recently, however, we (24) and others (25–28) have described solutions to these problems and have shown that appropriate use of the rapid filtration technique should permit the detection of PCP receptors. Moreover, Quirion *et al.* (28) have described identical sites using both direct binding to slide-mounted brain sections and autoradiography.

Recent animal-behavioral studies show that cyclazocine-like opaites display PCP-like properties. In a discriminative stimulus test using rats trained to cyclazocine, Teal and Holtzman (29) found that, of 12 test compounds, ketocyclazocine, SKF 10,047, ethylketocyclazocine, PCP, ketamine, pentazocine, and levallorphan generalized to cyclazocine (the last two most weakly); morphine, nalorphine, amphetamine, mescaline, and *N*,*N*-diethyl-D-lysergamide were inactive. Discriminative effects of cyclazocine were only partially reversible by naloxone. In a discriminative stimulus paradigm utilizing rats trained to PCP, Holtzman (30) has found that animals trained to PCP generalize to cyclazocine, SKF 10,047 and cylorphan, whereas they do not generalize to nalorphine. The present study provides biochemical evidence for the suggestion by Teal and Holtzman (29) that cyclazocine effects in rats have both an opioid and a non-opioid (non-naloxone-reversible) component, the latter showing some qualities of PCP effects.

It should be noted that the downward curvature observed in the [^{3}H]cyclazocine Scatchard binding plot (Fig. 1B) might result either from interaction at two or more binding sites of different affinities or from negatively cooperative binding to a single site. Three pieces of evidence suggest that these data demonstrate the binding of cyclazocine to different sites. First, the relative potencies of a series of opiates in their abilities to inhibit 1.0 nM and 70 nM [^{3}H]cyclazocine are markedly different. Second, the dissociation and association rate constants for 2 nM and 80 nM [^{3}H]cyclazocine binding are different. Third, the sensitivity of [^{3}H]cyclazocine binding to sodium and to NEM inactivation at these two ligand concentrations is different.

The identity of the intermediate cyclazocine binding site demonstrated here has not yet been determined. The tempting conclusion that this is the κ-site is suggested by the rank order of potency of opiates in displacement of 11 nM [^{3}H]cyclazocine. The low potency observed for naloxone would then be somewhat surprising; it would be of interest to study the effects of a more specific antagonist. Alternatively, the biphasic [^{3}H]cyclazocine binding analysis observed in the presence of 10 μM PCP could be indicative of negatively cooperative binding to a single site. Direct binding studies with [^{3}H]ethylketocyclazocine or development of more highly selective κ ligands might prove useful in furthering these investigations.

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