THE BINDING OF κ - AND σ -OPIATES IN RAT BRAIN¹

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Received July 9, 1981; Revised December 17, 1981; Accepted January 19, 1982

Abstract

Detailed displacements of [³H]dihydromorphine by ketocyclazocine and SKF 10,047, [³H]ethylketocyclazocine by SKF 10,047, and [³H]SKF 10,047 by ketocyclazocine are all multiphasic, suggesting multiple binding sites. After treating brain tissue *in vitro* with naloxazone, all displacements lose the initial inhibition of ³H-ligand binding by low concentrations of unlabeled drugs. Together with Scatchard analysis of saturation experiments, these studies suggest a common site which binds μ -, κ -, and σ -opiates and enkephalins equally well and with highest affinity ($K_D < 1$ nM). The ability of unlabeled drugs to displace the low affinity binding of [³H]dihydromorphine ($K_D = 3$ nM), [³H]ethylketocyclazocine ($K_D = 4$ nM), [³H]SKF 10,047 ($K_D = 6$ nM), and D-Ala²-D-Leu⁵-[³H]enkephalin ($K_D = 5$ nM) remaining after treating tissue with naloxazone demonstrates unique pharmacological profiles for each. These results suggest the existence of distinct binding sites for κ - and σ -opiates which differ from those sites which selectively bind morphine (μ) and enkephalin (δ).

Following the classification *in vivo* of various groups of opiates according to their pharmacological effects (Martin et al., 1976), several attempts have been made to identify distinct receptors for the κ and the σ class of opiates, named after the prototypic drugs ketocyclazocine and SKF 10,047 (*N*-allylnormetazocine), respectively. Although several groups initially were unable to demonstrate these κ and σ sites (Hiller and Simon, 1979, 1980; Snyder and Goodman, 1980), a number of laboratories now have presented evidence strongly suggesting their presence, particularly of κ sites (Kosterlitz and Patterson, 1980; McLawhon et al., 1981; Chang et al., 1980, 1981; Pfeiffer and Hertz, 1981; Wood et al., 1980).

Earlier studies from our laboratory implied more than one [³H]ethylketocyclazocine site (Pasternak, 1980). Intensive investigations of high affinity [³H]ethylketocyclazocine binding suggested that it was the same as the high affinity [³H]dihydromorphine binding site and that this common high affinity site, the μ_1 site (Wolozin and Pasternak, 1981; Zhang et al., 1981), mediated the analgesic effects of ketocyclazocine (Pasternak, 1980) in ad-

dition to those of morphine (Pasternak et al., 1980a, b), the enkephalins, and β -endorphin (Zhang and Pasternak, 1981; Pasternak, 1981). These studies focused on high affinity [³H]ethylketocyclazocine binding and did not address in great detail its lower affinity binding. Since earlier studies demonstrated that selective binding of morphine to μ sites and enkephalins to δ sites reflects differences in their lower affinity binding sites (Zhang and Pasternak, 1981; Zhang et al., 1981; Wolozin and Pasternak, 1981), we investigated the binding interactions of ³H-labeled μ , κ , σ , and δ ligands and their displacement by a number of unlabeled drugs in an effort to examine the possibility of κ - and σ -selective sites.

Materials and Methods

[³H]Dihydromorphine, [³H]ethylketocyclazocine, ($\pm$)-[³H]SKF 10,047, D-Ala²-D-Leu⁵-[³H]enkephalin, and Formula 963 scintillation fluor were obtained from New England Nuclear Corp. (Boston, MA). D-Ala²-D-Leu⁵-Enkephalin was obtained from Peninsula Laboratories (San Carlos, CA), and ($\pm$)-SKF 10,047 was obtained from the National Institute of Drug Abuse. Male Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Wilmington, MA). Naloxazone was synthesized as previously reported (Pasternak and Hahn, 1980).

Membranes were prepared and binding experiments were performed as previously described (Pasternak et al., 1975). In brief, washed membrane preparations were incubated with the ³H-ligand for 60 min at 25°C and samples (1 to 2 ml with 20 mg of tissue/ml) were filtered over Whatman GF/B filters and counted. Assays were

¹We wish to thank Drs. David Ahern and Jerome B. Posner for their assistance in these studies and Dr. Robert Willette for the generous gift of SKF 10,047. This work was supported in part by Grant PDT 169 from the American Cancer Society and Grant DA02615 from the National Institute of Drug Abuse.

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conducted in triplicate and all values reported represent specific [^3H]opiate binding, the difference between binding in the presence and absence of 1 μM levallorphan. Naloxazone treatment also was performed as previously described (Childers and Pasternak, 1982). In this technique, tissue was incubated with nothing or naloxazone (2 μM) at 25°C for 30 min. The homogenate then was washed four times before binding assays. Each wash consisted of an incubation at 25°C for 15 min followed by centrifugation and resuspension of the pellet. This procedure effectively removes reversible ligands.

Results

Pharmacological properties of high affinity binding sites. Naloxazone, an irreversible inhibitor of opiate re-

ceptor binding, selectively blocks the high affinity binding component identified by Scatchard analysis of [^3H] dihydromorphine (Pasternak et al., 1980a, b; Zhang and Pasternak, 1981), [^3H]ethylketocyclazocine (Pasternak, 1980), [^3H]SKF 10,047 (Pasternak et al., 1981), and D-Ala²-D-Leu⁵-[^3H]enkephalin (Zhang and Pasternak, 1981; Hazum et al., 1981), suggesting that these ligands bind with the highest affinity to a common site. However, the possibility of separate sites with similar sensitivities to naloxazone cannot be excluded. In an effort to help answer this question, competitive displacement studies have been performed on [^3H]dihydromorphine binding by unlabeled SKF 10,047 and ketocyclazocine. The displacement of [^3H]dihydromorphine binding in control tissue by ketocyclazocine is biphasic (Fig. 1A). There is

Figure 1. Displacement of [^3H]dihydromorphine binding by ketocyclazocine and SKF 10,047. Brain membranes were prepared and treated with nothing (●) or naloxazone (2 μM ; ○). Binding assays with [^3H]dihydromorphine (1 nM) then were performed in the presence of (A) ketocyclazocine or (B) SKF 10,047. The values reported represent specific binding. Binding in the absence of a displacer in control tissue and naloxazone-treated tissue, respectively, was 2600 and 950 cpm for the ketocyclazocine experiment and 4700 and 1100 cpm for the SKF 10,047 experiment. The differences were due to the different specific activities of [^3H]dihydromorphine in the two studies. The experiment has been replicated three times with similar results.

an initial displacement by low concentrations of ketocyclazocine (<3 nM) and a second displacement requiring 50-fold greater concentrations. Treatment of the tissue with naloxazone eliminates the initial displacement of [3 H]dihydromorphine binding by ketocyclazocine. Similar results are seen using SKF 10,047 (Fig. 1B). Binding is inhibited in a biphasic manner in control tissue and the initial displacement is reduced markedly by naloxazone treatment.

Unlabeled SKF 10,047 also displaces [3 H]ethylketocyclazocine binding in a biphasic fashion. As with [3 H]

dihydromorphine binding, the initial inhibition by SKF 10,047 is eliminated in tissue treated with naloxazone (Fig. 2). In a reciprocal experiment, the initial inhibition of [3 H]SKF 10,047 binding by unlabeled ketocyclazocine also is eliminated in naloxazone-treated tissue (Fig. 3). Since the above displacement experiments and the results of Scatchard analysis both suggest that the naloxazone-sensitive binding of all of the 3 H-ligands tested represented a common site, we investigated the binding remaining after naloxazone treatment.

Pharmacological properties of low affinity binding

Figure 2. Displacement of [3 H]ethylketocyclazocine binding by SKF 10,047. Brain membranes were prepared and treated with nothing (●) or naloxazone (2 μ M; ○). Binding assays were performed with [3 H]ethylketocyclazocine (1 nM) and various concentrations of SKF 10,047. Specific binding in the absence of a displacer was 1300 and 480 cpm in control and naloxazone-treated tissue, respectively. The experiment was replicated three times with similar results.

Figure 3. Displacement of [3 H]SKF 10,047 binding by ketocyclazocine. Brain membranes were prepared and treated with nothing (●) or naloxazone (2 μ M; ○). Binding assays then were performed with [3 H]SKF 10,047 (1 nM) in the presence of various concentrations of ketocyclazocine. Specific binding in the absence of a displacer was 2600 and 680 cpm in control and naloxazone-treated tissue, respectively. The experiment has been replicated three times with similar results.

sites. The ability of a series of unlabeled opiates to inhibit the binding of a variety of ^3H -ligands in tissue whose high affinity (μ_1) binding was inhibited by naloxazone was studied next (Table I). Clearly, the pharmacological binding characteristics of each of the four ^3H -ligands are quite distinct, suggesting that each ^3H -ligand labels sites to which the others do not bind potently. These differences are best illustrated by the selective displacement of [^3H]dihydromorphine binding by morphine, [^3H]ethylketocyclazocine binding by ketocyclazocine, and D-Ala²-D-Leu⁵-[^3H]enkephalin binding by D-Ala²-D-Leu⁵-enkephalin. [^3H]SKF 10,047 binding is a little more difficult to interpret. Unlabeled SKF 10,047 inhibits both [^3H]SKF 10,047 and [^3H]ethylketocyclazocine binding equally well. However, ketocyclazocine's 5-fold greater potency for [^3H]ethylketocyclazocine binding does imply that [^3H]SKF 10,047 binding includes a site not labeled by [^3H]ethylketocyclazocine.

The major finding with most of the various opiates tested is their lack of specificity for a single site. In our studies, morphine, D-Ala²-D-Leu⁵-enkephalin, and ketocyclazocine are the most selective. Ethylketocyclazocine is far more potent than ketocyclazocine but inhibits [^3H]dihydromorphine binding almost as well as [^3H]ethylketocyclazocine. Nalorphine has a high affinity for [^3H]dihydromorphine binding but is also potent on the binding of the κ and σ ^3H -ligands. Pentazocine shows little specificity among the various opiates and cyclazocine does not even discriminate well between the opiates and enkephalins despite its high potency.

Regional distribution of opioid binding. Differences in the regional localization of μ and δ binding sites have been well established (Chang and Cuatrecasas, 1979; Goodman et al., 1980). We therefore investigated the

regional binding of μ , κ , σ , and δ ^3H -ligands (Table II). Despite the fact that this assay procedure measures both high and low affinity binding sites, we find significant differences in the relative amounts of binding for the different ^3H -ligands in several regions of the brain. The contrast between the low levels of D-Ala²-D-Leu⁵-[^3H]enkephalin and the high levels of [^3H]SKF 10,047 binding in the hypothalamus is the most dramatic, followed by differences in the levels in the frontal cortex where [^3H]SKF 10,047 and D-Ala²-D-Leu⁵-[^3H]enkephalin binding are about the same and those in the striatum where D-Ala²-D-Leu⁵-[^3H]enkephalin binding is lower than the others.

Discussion

Opiate receptor heterogeneity has been postulated from *in vitro* and *in vivo* approaches. Based on studies in the chronic spinal dog, Martin and co-workers (1976) divided opiates into three major classes and named them for prototypic drugs: μ (morphine), κ (ketocyclazocine), and σ (SKF 10,047; *N*-allylnormetazocine). Subsequent studies investigating the enkephalins (Lord et al., 1977; Chang and Cuatrecasas, 1979) added another class of receptors (δ receptors) which preferentially bind enkephalins better than morphine, in contrast to the μ sites which have a higher affinity for morphine than enkephalins. Biochemical demonstration of κ sites proved more difficult. Several groups were unable to show κ -specific binding (Hiller and Simon, 1979, 1980; Snyder and Goodman, 1980; Chang et al., 1980), but subsequent reports have suggested their existence (Kosterlitz and Patterson, 1980; Chang et al., 1981; Pfeiffer and Hertz, 1981; Pasternak et al., 1981; McLawhon et al., 1981). Most studies examined the inhibition of a series of ^3H -ligands repre-

TABLE I
Inhibition of opiate receptor binding in naloxazone-treated tissue

Rat brain membranes were prepared and treated with naloxazone (2 μM). Their binding was determined with each ^3H -ligand (2 nM) and six concentrations of unlabeled ligand. The IC_{50} values were determined by least square fit to a log probit analysis. The values in the table represent the means $\pm$ SEM of three separate IC_{50} determinations except for SKF 10,047 which was tested four times. Differences in the ability of the different unlabeled ligands to inhibit a ^3H -ligand were established by an analysis of variance. Specific tissue binding of the various ^3H -ligands after naloxazone treatment (mean $\pm$ SEM of 25 separate experiments) were 2550 $\pm$ 230 cpm for [^3H]dihydromorphine, 830 $\pm$ 60 cpm for [^3H]ethylketocyclazocine, 1430 $\pm$ 90 cpm for [^3H]SKF 10,047, and 940 $\pm$ 75 cpm for D-Ala²-D-Leu⁵-[^3H]enkephalin.

Drug	IC_{50}				Probability
	[^3H]Dihydromorphine	[^3H]Ethylketocyclazocine	[^3H]SKF 10,047	D-Ala ² -D-Leu ⁵ -[^3H]Enkephalin	
	nM				
Morphine	13.1 $\pm$ 2.8	82 $\pm$ 39	143 $\pm$ 39	276 $\pm$ 92	N.S. ^a
Ketocyclazocine	130 $\pm$ 36	37.6 $\pm$ 3.7	193 $\pm$ 41	155 $\pm$ 40	$F_{3,8} = 4.16$ $p < 0.01$
Ethylketocyclazocine	12.3 $\pm$ 3.6	11.9 $\pm$ 3.6	25.5 $\pm$ 8.7	18.3 $\pm$ 0.9	$F_{3,8} = 9.75$ N.S.
<i>N</i> -Allylnormetazocine (SKF 10,047)	38.8 $\pm$ 13	20.2 $\pm$ 7.2	24.5 $\pm$ 12	51.8 $\pm$ 24	$F_{3,8} = 1.63$ N.S.
Cyclazocine	6.4 $\pm$ 1.0	3.7 $\pm$ 0.9	4.5 $\pm$ 2.0	5.8 $\pm$ 0.6	$F_{3,12} = 0.88$ N.S.
Pentazocine	101 $\pm$ 26	89.8 $\pm$ 27.1	119 $\pm$ 19	178 $\pm$ 37	$F_{3,8} = 0.98$ N.S.
Nalorphine	7.4 $\pm$ 0.6	15.0 $\pm$ 1.3	16.0 $\pm$ 1.0	61.2 $\pm$ 5.6	$F_{3,8} = 1.96$ $p < 0.001$
D-Ala ² -D-Leu ⁵ -Enkephalin	323 $\pm$ 91	448 $\pm$ 43	371 $\pm$ 65	20.4 $\pm$ 2.6	$F_{3,8} = 70.6$ $p < 0.01$
					$F_{3,8} = 9.75$

^a N.S., not significant.

TABLE II
Regional distribution of opioid binding

Rat brains were dissected as follows: following removal of the cerebellum, the brainstem was transected from the superior aspect of the pons to immediately below the inferior colliculus and this area was considered the pons/medulla. The frontal cortex, hypothalamus, and corpus striatum were dissected. The thalamus and the remaining midbrain then were removed and together constitute the thalamus/midbrain. Each experiment required the dissection of five rat brains to obtain sufficient tissue to perform the binding experiments. Once dissected, the various brain regions, including an additional whole rat brain, were assayed together with the various ^3H -ligands (all at 1 nM). Note that each assay tube contained 2 ml with 20 mg of tissue/ml with the exception of hypothalamus and striatum. Within each experiment, the binding of each ^3H -ligand in each region then was compared to its binding to whole brain and expressed as a percentage ((binding in region/binding in whole brain) $\times$ 100). The results indicate the mean $\pm$ SEM of n separate experiments. The ratios of binding for the various ^3H -ligands in each region then were compared using an analysis of variance. The means $\pm$ SEM of specific whole brain binding in the five experiments were: [^3H]dihydromorphine, 5127 ± 667 cpm (1.8 ± 0.24 fmol/mg of tissue); [^3H]ethylketocyclazocine, 1446 ± 67 cpm (2.0 ± 0.09 fmol/mg of tissue); [^3H]SKF 10,047, 1432 ± 111 cpm (1.3 ± 0.09 fmol/mg of tissue); and D-Ala²-D-Leu⁵-[^3H]enkephalin, 2053 ± 436 cpm (1.7 ± 0.4 fmol/mg of tissue).

Region	[^3H]Dihydro-morphine	[^3H]Ethylketo-cyclazocine	[^3H]SKF 10,047	D-Ala ² -D-Leu ⁵ -[^3H]Enkephalin	Probability
Frontal cortex (4) ^a	71 $\pm$ 6	76 $\pm$ 5	104 $\pm$ 11	104 $\pm$ 10	$p < 0.05$ $F_{3, 12} = 4.74$
Striatum (4)	32 $\pm$ 1	40 $\pm$ 6	52 $\pm$ 4	35 $\pm$ 3	$p < 0.05$ $F_{3, 12} = 4.87$
Hypothalamus (3)	15 $\pm$ 1	10 $\pm$ 1	23 $\pm$ 5	1 $\pm$ 1	$p < 0.001$ $F_{3, 8} = 15.15$
Thalamus/midbrain (4)	116 $\pm$ 15	113 $\pm$ 24	125 $\pm$ 19	71 $\pm$ 13	N.S. ^b $F_{3, 12} = 2.53$
Pons/medulla (5)	54 $\pm$ 5	64 $\pm$ 14	75 $\pm$ 6	42 $\pm$ 6	N.S. $F_{3, 12} = 2.59$
Spinal cord (3)	45 $\pm$ 4	53 $\pm$ 3	46 $\pm$ 12	45 $\pm$ 13	N.S. $F_{3, 8} = 0.19$

^a Number of determinations.

^b N.S., not significant.

senting the various classes of drugs. Although multiphase displacement curves were reported by most laboratories, only one subjected these displacements to sophisticated computer analysis (Pfeiffer and Hertz, 1981). While these studies agreed on the existence of a site which binds benzomorphans preferentially, some questions remained about the existence of both κ and σ sites.

Our results suggest distinct binding sites for [^3H]ethylketocyclazocine and [^3H]SKF 10,047 which may correspond to κ and σ sites. The ability of naloxazone to block selectively the high affinity competitive interactions between the different classes of drugs (Figs. 1, 2, and 3), coupled with its selective inhibition of high affinity sites for the four ^3H -ligands demonstrated with Scatchard analysis (Pasternak et al., 1980a, b; Pasternak, 1980; Hazum et al., 1981; Zhang and Pasternak, 1981; Pasternak et al., 1981), suggests a common high affinity binding site for all. This site, corresponding to the previously described μ_1 site (Wolozin and Pasternak, 1981; Zhang et al., 1981), has been implicated in the analgesic mechanisms of all four classes of drugs (Pasternak et al., 1980a, b, 1981; Pasternak, 1980, 1981; Zhang and Pasternak, 1981). The binding characteristics of the low affinity [^3H]dihydromorphine binding (μ_2 site; $K_D = 3$ nM) and the δ site, corresponding to low affinity D-Ala²-D-Leu⁵-[^3H]enkephalin binding ($K_D = 5$ nM), are distinct from both each other and from the low affinity [^3H]ethylketocyclazocine ($K_D = 4$ nM) and [^3H]SKF 10,047 ($K_D = 6$ nM) sites. Ketocyclazocine's selectivity for the low affinity [^3H]ethylketocyclazocine binding compared to [^3H]SKF 10,047 binding implies that these two ^3H -ligands do not label the same sites and suggests separate κ - and σ -selective binding sites.

The differences in the regional localization of the binding of the ^3H -ligands also are suggestive of separate binding sites. Most interesting is the hypothalamus, which has little δ binding. However, this type of approach has many limitations and definitive studies will require autoradiographical techniques.

Although the ability of unlabeled drugs to inhibit ^3H -ligand binding in cross-displacement studies using naloxazone-treated tissue strongly suggests selective binding sites for these four ^3H -ligands, one cannot automatically assign low affinity [^3H]ethylketocyclazocine and [^3H]SKF 10,047 binding as the κ and σ receptors, respectively. Even after simplifying the binding by blocking the high affinity, or μ_1 , sites, the various unlabeled drugs are not very selective, particularly those binding to putative κ and σ sites. The binding remaining after naloxazone treatment probably reflects more than one site. Therefore, the IC_{50} values most likely reflect an average value for several different types of sites. The relatively shallow displacement by ketocyclazocine of [^3H]SKF 10,047 binding in the naloxazone-treated tissue also may reflect this binding heterogeneity. Discriminating between these different sites remains difficult and may require the development of more selective ligands. Alternatively, one could study tissues which possess fewer types of sites. Thus, the presence of benzomorphan-selective sites in neuroblastoma $\times$ brain hybrid cell lines (McLawhon et al., 1981) may prove helpful in their characterization.

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