MOLECULAR PHARMACOLOGY, 21:266–271

Discrimination of Three Opiate Receptor Binding Sites with the Use of a Computerized Curve-Fitting Technique

ANDREAS PFEIFFER[1] AND ALBERT HERZ

Department of Neuropharmacology, Max Planck Institute for Psychiatry, 8 Muenchen 40, Federal Republic of Germany

Received August 4, 1981; Accepted October 30, 1981

SUMMARY

The presence of different types of opiate binding sites was investigated with the use of a computerized, weighted, nonlinear least-squares regression program. The experimental data were obtained from four groups. Each of three labeled opiate ligands was displaced using each of the same unlabeled ligands. The resulting nine different ligand combinations of each group were evaluated by use of a curve-fitting program. The four groups consisted of the κ ligand ethylketocyclazocine, the σ ligand SKF 10047, and the oripavine derivatives etorphine and diprenorphine, each in conjunction with the δ opiate receptor ligand (D-Ala^2,D-Leu^5)-enkephalin and the μ opiate receptor ligand dihydromorphine. The binding model which best fitted each of the four groups suggested the existence of three different binding sites in the rat brain homogenate. Two of these sites conform to the previously described μ and δ sites. A third site (R_3) displayed high affinity for ethylketocyclazocine, SKF 10047, etorphine, and diprenorphine but very low affinity for dihydromorphine and [D-Ala^2,D-Leu^5]enkephalin. Naloxone, cyclazocine, and dynorphin-(1–13) had high affinity for R_3. Behavioral data support the interpretation that the R_3 site may represent a κ site at which SKF 10047 acts antagonistically.

INTRODUCTION

Multiple classes of opiate receptors have been suggested by both behavioral and radioligand binding studies. However, the types of opiate receptors suggested by use of the different techniques are partially discrepant. At present, three types of opiate receptors are distinguished *in vivo* according to the different behavioral syndromes produced by the respective prototype agonists (1–3), i.e., morphine at μ receptors, ethylketocyclazocine at κ receptors, and SKF 10047 at σ receptors. On the other hand, binding studies have succeeded in identifying two distinct types of opiate binding sites, termed μ and δ, with morphine and DADL[2] as their respective prototype ligands (4–7). Various investigations have failed to establish the presence of putative κ or σ sites in the rat brain (6–10). However, Kosterlitz *et al.* (11) provided some evidence for κ binding sites in guinea pig brain. Previous studies also reported a high affinity of ethylketocyclazocine and SKF 10047 for μ and δ sites (6, 7, 11–13). Thus, binding of these ligands to putative κ or σ sites should occur at a binding component additional to μ and δ sites. A resolution of displacement or binding isotherms into three components is difficult to achieve by use of conventional techniques. Computerized, nonlinear, curve-fitting techniques have proven of value in the study of luteinizing hormone-releasing hormone (14) and *beta*-adrenergic receptor binding (15). Therefore, in previous studies (12, 13), a combination of displacement experiments performed with mutual combinations of dihydromorphine, DADL, ethylketocyclazocine, and SKF 10047 and analysis of the experimental data by computerized, weighted, least-squares nonlinear regression curve-fitting techniques as developed by Munson and Rodbard (16) was used to resolve the various binding components of ethylketocyclazocine and SKF 10047. This permitted a distinction of three types of opiate binding sites in various areas of rat brain (13). The present study was carried out to evaluate the application of this technique for the distinction of multiple opiate binding sites in more detail. Moreover, the newly demonstrated binding site, termed preliminarily R_3, is further characterized with regard to other opiate and opioid ligands.

MATERIALS AND METHODS

Data analysis. Computer resolution of the displacement curves was performed using the "ligand" program as described by Munson and Rodbard (16). The weighted, nonlinear, least-squares regression curve-fitting program constructs models of binding according to the law of mass action for an interaction of multiple ligands with multiple binding sites. The computerized algorithm aims at reducing the deviation of the model of binding from the experimental data, with the experimental points being weighted according to the reciprocal of the predicted variance. The program permits simultaneous analysis of

This work was supported by the Deutsche Forschungsgemeinschaft.

[1] Present address, Laboratory of Clinical Science, National Institute of Mental Health, Bethesda, Md. 20205.

[2] The abbreviation used is: DADL, (D-Ala^2,D-Leu^5)-enkephalin.

0026-895X/82/020266-06$02.00/0

multiple displacement curves obtained with various combinations of labeled and unlabeled ligands and provides parameter estimates (± approximate standard error) for the binding affinities and capacities of the ligands. The standard error values indicated in the tables were calculated as proposed by Munson and Rodbard (14) from the approximate values. Nonspecific binding was treated as a fitted parameter. Binding models assuming three sites, two sites, or one site can be tested and compared statistically by an *F*-test incorporated in the program. A fit of the data assuming two or three binding sites was accepted as appropriate only if the fit was significantly improved over the fit assuming one site less. The level of significance chosen was $p < 0.01$.

For binding assays, rat brain homogenate obtained from whole brain minus cerebellum was washed three times in 5 mM Tris buffer (pH 7.4) and sedimented at 40,000 × *g* for 20 min between the washes. After dilution (1:100) in Tris-Krebs-Ringer buffer [composition (millimolar), NaCl, 118; $CaCl_2$, 2.54; $MgSO_4$, 1.2; KCl, 4.75; Tris, 50 (pH 7.4)], 2-ml portions were incubated at 28° for 35 min with 12–17 concentrations of the unlabeled displacer and 0.1–1 nM labeled ligand. Bound ligand was separated from free ligand by filtration over GFC glass-fiber discs (Whatman). Specific binding, determined by subtraction of binding in the presence of 10 μM unlabeled ligand from the total radioactivity retained on the filters, varied between 70% and 93%.

Materials. [^{3}H]dihydromorphine (73.2 Ci/mmole), [^{3}H]diprenorphine (9.1 Ci/mmole), [^{3}H]etorphine (34 Ci/mmole), and [^{3}H]DADL (42 Ci/mmole) were purchased from Amersham Buchler (Braunschweig, Germany), and [^{3}H]ethylketocyclazocine (15 Ci/mmole) and [^{3}H]SKF 10047 (44 Ci/mmole) were obtained from New England Nuclear Corporation (Dreieich, Germany). Unlabeled substances were obtained as follows: ethylketocyclazocine methanesulfonate (generously supplied by Sterling-Winthrop Research Institute, Rensselaer, N. Y.), SKF 10047 or *N*-allyl-normetazocine (Addiction Research Center, Lexington, Ky.), DADL and dynorphin-1–13 (Bachem AG, Bubendorf, Switzerland), naloxone (Endo Laboratories, Garden City, N. Y.), etorphine (Reckitt and Colman, Hull, England), sufentanyl (Janssen Pharmaceutica, Beerse, Belgium), and dihydromorphine (Merck, Darmstadt, Germany). All labeled ligands were purified by reverse-phase high-pressure liquid chromatography if necessary, which was usually the case for [^{3}H]dihydromorphine, [^{3}H]ethylketocyclazocine, [^{3}H]DADL, and [^{3}H]etorphine.

RESULTS

The interaction of ethylketocyclazocine, SKF 10047, dihydromorphine, and DADL with opiate binding sites was investigated by measuring the binding of each of the ligands to rat brain membrane preparations by means of homologous and heterologous displacement experiments. Parameter estimates of binding capacities and of equilibrium dissociation constants for the four opiate ligands were derived by use of the curve-fitting program. Initially, curves obtained from displacement experiments performed with two ligands were analyzed simultaneously (i.e., the two homologous and two heterologous displacement curves of each of the combinations) (Table 1). This type of analysis is termed "two-ligand fit."

Dihydromorphine displaced [^{3}H]dihydromorphine with a steep slope (Fig. 1). Computer estimates indicated an affinity of 2.2 ± 0.3 nM (K_d) and a capacity of 4.75 ± 0.3 pmoles/g of tissue. Dihydromorphine displaced [^{3}H]

TABLE 1

Parameter estimates of binding capacity and K_d for ethylketocyclazocine, SKF 10047, dihydromorphine, and DADL (two-ligand fits)

Results were obtained by simultaneous computerized curve fitting of the experimental data, obtained by displacement experiments performed with two ligands in the four possible combinations of labeled ligand and unlabeled displacer, i.e., ^{3}H-A versus A, ^{3}H-A versus B, ^{3}H-B versus A, and ^{3}H-B versus B. Two to four independent experiments were performed with each of the combinations. Eight to twelve curves were analyzed simultaneously. The standard errors were estimated by the program and include the among-experiment variations. The statistical values, as compared with binding models assuming one site less, were as follows: A, $DF = 112$, $F = 252$, $p < 0.01$; B, $DF = 84$, $F = 26.8$, $p < 0.01$; C, $DF = 85$, $F = 34$, $p < 0.01$; D, $DF = 94$, $F = 61.5$, $p < 0.01$; E, $DF = 76$, $F = 31$, $p < 0.01$.

Ligand combination	Binding capacity	Apparent dissociation constant (K_d)			
		Dihydromorphine	DADL	SKF 10047	Ethylketocyclazocine
	pmoles/g tissue	*nM*	*nM*	*nM*	*nM*
A. DADL and dihydromorphine	6.3 ± 0.6	2.6 ± 0.3	3.6 ± 0.3		
	4.6 ± 1.6	311 ± 47	2.2 ± 0.9		
B. Ethylketocyclazocine and dihydromorphine	46 ± 0.7	2.7 ± 0.5			1.6 ± 0.2
	10.4 ± 3.7	1,490 ± 814			9.9 ± 4.3
C. SKF 10047 and dihydromorphine	6.8 ± 1.1	2.4 ± 0.5		2.6 ± 0.5	
	14 ± 7.0	345 ± 105		13 ± 6.9	
D. SKF 10047 and DADL	7.0 ± 1.3		4.0 ± 0.7	4.8 ± 0.9	
	3.9 ± 2.7		2.5 ± 1.9	40.8 ± 13	
	2.7 ± 0.5		1,280 ± 520	4.8 ± 1.0	
E. Ethylketocyclazocine and DADL	9 ± 1.1		4.2 ± 0.7		3.7 ± 0.4
	3.7 ± 2.7		9,640 ± 5,030		4.6 ± 6.2

DADL, [^{3}H]ethylketocyclazocine, and [^{3}H]SKF 10047 with shallow slopes (Figs 1, 2, and 3), and there was 95–100% displacement at 10 μM. In order to fit these curves satisfactorily, the assumption of two binding components with which dihydromorphine interacted with approximately 100-fold differences in K_d values was required. In the two-ligand fit of dihydromorphine with DADL, the K_d value of DADL to the high-affinity component of dihydromorphine binding appeared to be slightly lower than that of dihydromorphine (Table 1). The apparent K_d value of DADL for the second site was about 2 nM, whereas the K_d value of dihydromorphine was more than 100-fold greater. These two sites conform well to the established μ and δ sites (4–7); therefore the term μ site is used subsequently for the binding component which is labeled by dihydromorphine with a K_d value in the nanomolar range, and the term δ site is used to indicate the binding component labeled by DADL with a K_d value in the nanomolar range whereas dihydromorphine displays a low apparent affinity to this site. Both ethylketocyclazocine and SKF 10047 interacted with the μ site with apparent nanomolar K_d values (Table 1). The low-affinity component of dihydromorphine binding that was apparent when deriving parameter estimates from the two-ligand fits obtained with combinations of either SKF 10047 or ethylketocyclazocine together with dihydromorphine was of greater capacity than the δ site described above. Ethylketocyclazocine and SKF 10047 had apparent K_d values of about 10 nM for this component. Displacement of [^{3}H]SKF 10047 by the μ agonist sufentanyl (17) permitted a distinction of two binding components displaying high and low affinities for sufentanyl, as was also observed before using dihydromorphine (Tables 1 and 3; Figs. 2 and 5).

DADL displaced [^{3}H]dihydromorphine and [^{3}H] DADL with steep slopes (Fig. 1). The shapes of displacement isotherms of DADL against [^{3}H]ethylketocyclazocine or [^{3}H]SKF 10047 were markedly different. Most of the radiolabeled ligand was displaced at low concentrations of DADL with a steep slope which then flattened at higher concentrations of the displacer (Figs. 2 and 3). At 10 μM, displacement was 85–95% complete. The two-ligand fit obtained with DADL and SKF 10047 was best represented by a model assuming three binding sites. This resulted from the fact that SKF 10047 displayed approximately 10-fold differences in K_d values to two apparent binding sites of DADL high-affinity binding, whereas DADL displayed more than 100-fold differences in affinity for the binding sites labeled by SKF 10047 (Table 1). Although the experimental data obtained with ethylketocyclazocine and DADL fitted a three-site model significantly better ($DF = 73$, $F = 5.3$, $p < 0.01$), this assumption resulted in large standard errors. The two-site fit was therefore preferred (Table 1), suggesting a large high-affinity site of both ligands and a second site of about 3–4 pmoles/g of tissue for which ethylketocyclazocine displayed high affinity whereas DADL displayed low affinity. Figure 4 shows a comparison of displacement curves of DADL and dihydromorphine

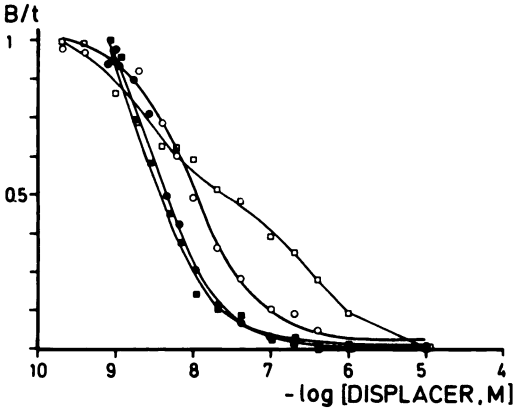

FIG. 1. *Displacement curve of unlabeled DADL against [^{3}H] DADL (●) and [^{3}H]dihydromorphine (○) and of unlabeled dihydromorphine against [^{3}H]dihydromorphine (■) and [^{3}H]DADL (□)*

The fraction of ^{3}H label retained on the filters after subtraction of nonspecific binding is plotted on the ordinate. The abscissa shows the total ligand concentration. The line was obtained from the weighted, least-squares, nonlinear regression program. The data shown are from one representative experiment which was replicated three times.

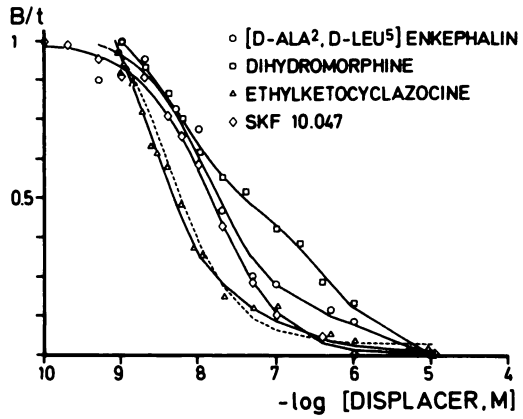

FIG. 2. *Displacement curves of labeled ethylketocyclazocine by unlabeled DADL, dihydromorphine, SKF 10047, and ethylketocyclazocine*

The *solid lines* for ethylketocyclazocine and SKF 10047 correspond to the data in Table 3, whereas the *interrupted line* indicates the fit of a one-site model. The other curves correspond to the data in Table 1. For further explanations see legend to Fig. 1.

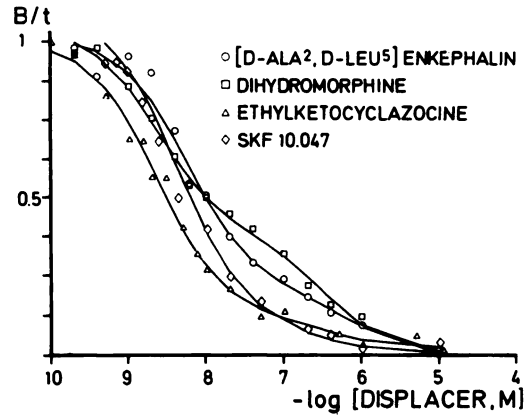

FIG. 3. *Displacement of labeled SKF 10047 by DADL, dihydromorphine, ethylketocyclazocine, and SKF 10047*

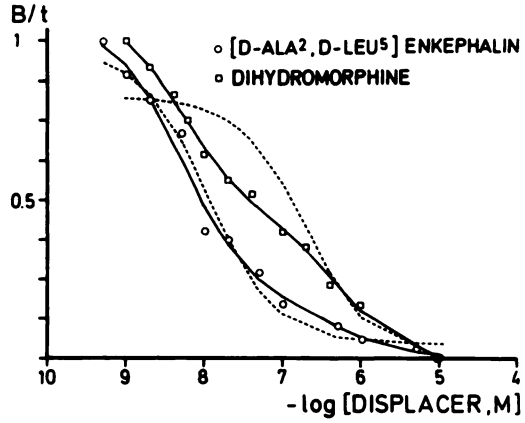

FIG. 4. *Comparison of fits assuming one binding site (interrupted line) or two binding sites for DADL and dihydromorphine against labeled ethylketocyclazocine*

The one-site binding models for DADL and dihydromorphine were significantly worse-fitted than the two-site models. The parameter estimates shown in Table 1 correspond to the *solid lines*.

against [^{3}H]ethylketocyclazocine assuming either one (– – –) or two binding components. This comparison clearly shows the better fit of a two-site model.

In order to obtain a better resolution of the binding components, data obtained using combinations of either ethylketocyclazocine or SKF 10047 together with DADL and dihydromorphine were simultaneously evaluated by the curve-fitting program. Thus, curves obtained using nine different ligand combinations of the three ligands were simultaneously modeled. This approach permitted a resolution into three different binding components. One site was labeled with nanomolar apparent K_d values by all ligands (R_1 in Table 2). A second component displayed high affinity for DADL, intermediate affinity for ethylketocyclazocine and SKF 10047, and low affinity for dihydromorphine (R_2 in Table 2). The third component was labeled with relatively high affinity by ethylketocyclazocine and SKF 10047, whereas dihydromorphine and DADL displayed very low affinity. Binding affinities of the oripavine derivative etorphine, which was shown to displace completely [^{3}H]ethylketocyclazocine with high affinity (11), and of the oripavine opiate antagonist diprenorphine were analyzed in a similar fashion. This also permitted the differentiation of three distinct binding sites, the capacities and affinities of which are in agreement with the data reported above (Table 2).

To establish that ethylketocyclazocine and SKF 10047 bind to the same sites, SKF 10047 was added in competition for [^{3}H]ethylketocyclazocine and ethylketocyclazo-

TABLE 2

Parameter estimates of binding capacity and K_d for ethylketocyclazocine, SKF 10047, dihydromorphine, DADL, diprenorphine, and etorphine (three-ligand fits)

Results were obtained by simultaneous evaluation of the data obtained using three different labeled and unlabeled ligands indicated in A–D, i.e., nine different ligand combinations were evaluated together. The experimental data regarding combinations of dihydromorphine and DADL were identical in A–D. A three-site model of binding represented the experimental data of A–D significantly better ($p < 0.01$), with *DF* between 180 and 310 and the following *F* values: A = 15.3, B = 29, C = 22.2, and D = 30.8.

Site	Binding capacity	K_d		
		Dihydromorphine	DADL	Ethylketocyclazocine
	pmoles/g tissue	*nM*	*nM*	*nM*
A. R_1	4.1 ± 0.6	2 ± 0.5	3.9 ± 0.8	1.1 ± 0.2
R_2	3.4 ± 1.6	233 ± 72	3.3 ± 1.6	12 ± 3.6
R_3	3.4 ± 2	760 ± 540	1,450 ± 1,230	3.1 ± 1.8
		Dihydromorphine	DADL	SKF 10047
B. R_1	5.6 ± 0.5	2.5 ± 0.31	3.8 ± 0.4	3.1 ± 0.3
R_2	5.0 ± 1.4	253 ± 47	3.1 ± 0.8	29 ± 3.7
R_3	6.5 ± .0	521 ± 134	945 ± 339	13 ± 6.1
		Dihydromorphine	DADL	Diprenorphine
C. R_1	4.5 ± 0.7	2 ± 0.25	3.3 ± 0.4	0.26 ± 0.03
R_2	3.5 ± 0.8	301 ± 44	2.8 ± 0.7	0.26 ± 0.03
R_3	6.0 ± 1.8	288 ± 54	549 ± 260	0.26 ± 0.03
		Dihydromorphine	DADL	Etorphine
D. R_1	4.3 ± 0.6	1.6 ± 0.26	3.3 ± 0.41	0.11 ± 0.024
R_2	3.9 ± 1.4	274 ± 42	2.8 ± 0.8	1.0 ± 0.7
R_3	5.5 ± 1.6	204 ± 92	685 ± 292	0.66 ± 0.4

TABLE 3

Parameter estimates of binding capacity and K_d for naloxone and sufentanyl

Results were obtained by simultaneous analysis of data obtained by displacement of [^{3}H]ethylketocyclazocine, [^{3}H]SKF 10047, and [^{3}H]dihydromorphine by naloxone together with data obtained by displacement of the labeled ligands by the homologous displacer and of [^{3}H]ethylketocyclazocine and [^{3}H]SKF 10047 by dihydromorphine. The three-site model (A) was better fitted (*DF* = 182, *F* = 14.9, $p < 0.01$) than a two-site model. In B, results were obtained by displacement of [^{3}H]SKF 10047 by sufentanyl (*DF* = 103, *F* = 34.5, $p < 0.01$, better than a fit assuming one binding site).

Site	Binding capacity	K_d			
		SKF 10047	Ethylketocyclazocine	Dihydromorphine	Naloxone
	pmoles/g tissue	*nM*	*nM*	*nM*	*nM*
A. R_1	5.7 ± 1.4	3.3 ± 0.4	1.66 ± 0.3	1.8 ± 0.2	1.6 ± 0.1
R_2	11 ± 1.9	12.4 ± 2.6	6.2 ± 3.5	179 ± 30	7.2 ± 0.95
R_L[a]	125 ± 58	1,770 ± 1,230	190 ± 87	>100,000	12,400 ± 6,350
		SKF 10047	Sufentanyl		
B. R_1	5.15 ± 1.25	3.5 ± 0.74	0.6 ± 0.09		
R_2	14.7 ± 5.8	30 ± 14	712 ± 267		

[a] Ethylketocyclazocine and SKF 10047 displayed low affinity to this site, which differs from R_3 in Table 2.

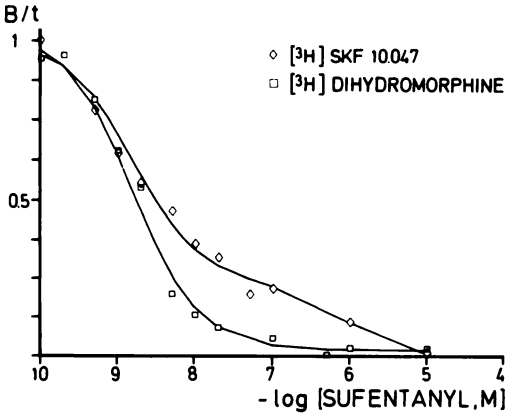

FIG. 5. *Displacement of labeled dihydromorphine and SKF 10047 by sufentanyl*

The curves shown correspond to the parameters indicated in Table 3.

cine in competition for [^{3}H]SKF 10047. These experiments were fitted satisfactorily by assuming one binding site of approximately 15 ± 2 pmoles for which ethylketocyclazocine had an affinity of 3 ± 0.3 nM and SKF 10047 an affinity of 8.6 ± 0.9 nM (K_d). However, a significantly better fit was obtained by assuming an additional low-affinity, high-capacity site to which ethylketocyclazocine and SKF 10047 displayed affinities of less than 100 nM (K_d) (DF 104, $F = 11$, $p < 0.01$). This site relied on points in the lower concentration range of the displacer (Fig. 2: ——, the two-site model; - - -, the one-site model) and could not be determined accurately. The binding of naloxone was assessed using displacement of [^{3}H]ethylketocyclazocine, [^{3}H]SKF 10047, and [^{3}H] dihydromorphine. Curve fitting of these data indicated three apparent binding components, one with high affinity for naloxone, which was also labeled with high affinity by dihydromorphine, and a second site to which naloxone displayed approximately a 10-fold greater K_d value. An additional binding component was labeled by ethylketocyclazocine and SKF 10047 with affinities of approximately 0.2 and 1.7 μM (K_d) and by naloxone with an affinity of 12 μM (K_d); dihydromorphine did not bind to this component (Table 3). In contrast to naloxone, cyclazocine displaced the total binding of [^{3}H]ethylketocyclazocine with an affinity of 1.25 ± 0.5 nM (K_d).

Interestingly, the 1–13 fragment of dynorphin (18) was also a potent displacer of [^{3}H]ethylketocyclazocine binding. In order to evaluate the interaction of dynorphin-(1–13) with opiate binding sites, displacement experiments against [^{3}H]ethylketocyclazocine, [^{3}H]dihydromorphine, and [^{3}H]DADL were performed. Since specific inhibitors of dynorphin-degrading enzymes are presently not established, the experiments were performed at 28° for 30 min in the absence of protease inhibitors. Computer fitting of these data (six curves), assuming the binding parameters for ethylketocyclazocine, dihydromorphine, and DADL as established above (Table 2), suggested that dynorphin-(1–13) had affinities of 6.5 ± 0.6 nM (K_d) for the μ site, 60 ± 10 nM for the δ site, and 1.4 ± 1.1 nM for R_3.

DISCUSSION

Computerized analysis of binding data obtained by heterologous displacement experiments of the μ and δ ligands dihydromorphine and DADL in combination with ethylketocyclazocine, SKF 10047, etorphine, or diprenorphine enabled a differentiation of three apparent opiate binding sites. Two of these correspond to the established μ and δ sites characterized by a high-affinity interaction with dihydromorphine and DADL, respectively (4–7). A third site (R_3) interacted with apparent nanomolar K_d values with ethylketocyclazocine, SKF 10047, etorphine, and diprenorphine, whereas dihydromorphine and DADL had affinities of approximately 0.3 and 0.8 μM (K_d). Evidence for R_3 was acquired by "substracting" μ and δ binding sites from the total binding capacity of ethylketocyclazocine, SKF 10047, etorphine, or diprenorphine by using computerized modeling techniques of displacement curves. Quantitative estimates of binding parameters, derived from mutual displacement experiments performed with two ligands (Table 1), indicated that the high-affinity component of the displacement curve of DADL against ethylketocyclazocine and SKF 10047 was of higher capacity than the high affinity component of the respective curves performed using dihydromorphine. Conversely, the low-affinity component, apparent when displacing ethylketocyclazocine and SKF 10047 using dihydromorphine, had about twice the capacity of the low-affinity component apparent with DADL against ethylketocyclazocine and SKF 10047. This finding confirms that binding capacities and affinities as assessed with two ligands are compatible with the three-site model of opiate binding. A good resolution into three distinct components was achieved only by simultaneous evaluation of the μ and δ ligands together with one of the higher-capacity ligands, i.e., ethylketocyclazocine, SKF 10047, etorphine, or diprenorphine. Remarkably, both ethylketocyclazocine and SKF 10047 displayed highest affinity to the μ site, identified by a high affinity for dihydromorphine, but approximately 3-fold greater K_d values for R_3 and approximately 10-fold greater K_d values for the δ site identified by a high affinity for DADL and a low affinity for dihydromorphine. In contrast, the oripavine opiate antagonist diprenorphine displayed about equal affinities to all types of opiate binding sites.

Ethylketocyclazocine and SKF 10047 displaced each other completely, suggesting an interaction with identical sites. Moreover, naloxone also displaced ethylketocyclazocine and SKF 10047 binding almost completely at concentrations of less than 100 nM, suggesting an interaction with all three opiate binding sites. In agreement with results of Lord *et al.* (4), naloxone displayed higher affinity for the μ site than for the δ site and also for R_3 (summarized as R_2 in Table 3A). Experiments performed with ethylketocyclazocine, SKF 10047, and naloxone were evidential of an additional low-affinity binding component of ethylketocyclazocine and SKF 10047, for which naloxone displayed extremely low affinity and dihydromorphine displayed negligible affinity (termed R_L in Table 3 to avoid confusion with R_3). It may therefore not represent an opiate binding site. Unfortunately, this component could not be determined accurately, and requires demonstration using a more selective or higher-affinity ligand. Speculatively, this binding component could correspond to the low-affinity binding of ethylketocyclazocine and SKF 10047 to a phencyclidine site, as proposed

by Zukin and Zukin (19). Very recently, Kosterlitz *et al.* (20) and Chang *et al.* (21) provided evidence for binding sites in brain displaying a high affinity for ethylketocyclazocine and other benzomorphans but very low affinity for dihydromorphine and DADL. These sites, termed κ by Kosterlitz *et al.* (20) and benzomorphan by Chang *et al.* (21), appear to correspond to R_3 indicated by this study. The κ sites demonstrated in guinea pig brain appear to display approximately 5-fold higher affinity for benzomorphans (20) than the respective sites in rat brain (12, 13, 21), and may moreover represent a more important fraction of the total opiate-binding capacity present.

Although three distinct opiate binding sites may be differentiated by binding experiments, it is difficult to reconcile these data with results obtained *in vivo*. A surprising finding of this study was that both ethylketocyclazocine and SKF 10047, the prototype ligands at κ and σ receptors, respectively (1), labeled R_3 with high affinity. Behavioral effects of ethylketocyclazocine and SKF 10047 differ greatly (1, 2, 3, 22). In most studies performed with rats, the agonistic-like actions of SKF 10047 were poorly naloxone-antagonizable (2, 3, 22), suggesting that they are not mediated by opiate receptors. In contrast, ethylketocyclazocine elicits naloxone-reversible effects in rodents which appear not to be mediated by κ receptors (17, 22, 23). Analgesia (3) and narcotic drug discrimination studies (24, 25) indicate that SKF 10047 is an antagonist of ethylketocyclazocine in rats. R_3 thus may correspond to a κ receptor site at which SKF 10047 is an antagonist, in addition to its established antagonistic effects at μ receptors (1). However, it remains to be explained why ethylketocyclazocine has no agonistic or antagonistic actions at μ receptors, despite its high affinity for this binding site. Most of the studies suggesting that ethylketocyclazocine displays no activity at μ receptors were performed using morphine-dependent animals (1, 17, 23), whereas one study indicated that ethylketocyclazocine elicits analgesia at high-affinity morphine sites (26). Thus it is presently not clear whether or not ethylketocyclazocine is an agonist at μ receptors in nondependent animals. Other effects of ethylketocyclazocine appear to be mediated by a distinct class of receptor sites (1, 2, 17, 22, 24, 25) which could correspond to the R_3 site demonstrated in this study.

With regard to a possible endogenous ligand at R_3, dynorphin was shown to display a high affinity for this site. Since degradation of dynorphin-(1–13) probably occurred under the experimental conditions used, these preliminary values in each case may be lower than the actual ones. The possibility that degradation products of dynorphin-(1–13) could possess a significant affinity for R_3 cannot be completely excluded. However, this agrees well with the high affinity of dynorphin-(1–13) for κ sites in the guinea pig ileum (17) and may indicate some similarity with the central nervous system R_3 site. It should be noted that β endorphin sites which display high affinity for cyclazocine and diprenorphine but low affinity for various μ and δ ligands have been demonstrated by Law *et al.* (27). Thus, a characterization of R_3 with regard to β endorphin appears desirable.

ACKNOWLEDGMENTS

The authors wish to thank P. J. Munson and D. Rodbard for providing the curve-fitting program. They acknowledge the help of H.-P. Rohde and L. V. Lindern and are indebted to M. Wuester and G. T. Shearman for helpful discussion and correction of the manuscript.

REFERENCES

1. Martin, W. R., C. G. Eades, J. A. Thompson, R. E. Huppler, and P. E. Gilbert. The effects of morphine and nalorphine like drugs in the nondependent and morphine dependent chronic spinal dog. *J. Pharmacol. Exp. Ther.* **197**:517–532 (1976).
2. Cowan, A. Simple in vivo tests that differentiate prototype agonists at opiate receptors. *Life Sci.* **28**:1559–1570 (1981).
3. Iwamoto, E. T. Locomotor activity and antinociception after putative *mu*, *kappa* and *sigma* opioid receptor agonists in the rat: influence of dopaminergic agonists and antagonists. *J. Pharmacol. Exp. Ther.* **217**:451–460 (1980).
4. Lord, J. A. H., A. A. Waterfield, J. Hughes, and H. W. Kosterlitz. Endogenous opioid peptides: multiple agonists and receptors. *Nature (Lond.)* **267**:495–499, (1977).
5. Gillan, M. G. C., H. W. Kosterlitz, and S. J. Paterson. Comparison of the binding characteristics of tritiated opiates and opioid peptides. *Br. J. Pharmacol.* **70**:481–490 (1980).
6. Chang, K.-J., E. Hazum, and P. Cuatrecasas. Possible role of distinct morphine and enkephalin receptors in mediating actions of benzomorphan drugs (putative κ and σ agonists). *Proc. Natl. Acad. Sci. U. S. A.* **77**:4469–4473 (1980).
7. Snyder, S. H., and R. R. Goodman. Neurotransmitter receptors. *J. Neurochem.* **35**:5–15 (1980).
8. Harris, D. W., and V. H. Sethy. High affinity binding of [^{3}H]ethylketocyclazocine to rat brain homogenate. *Eur. J. Pharmacol.* **66**:121–123 (1980).
9. Hiller, J. M., and Simon, E. J. [^{3}H]Ethylketocyclazocine binding: lack of evidence for a seperate kappa receptor in rat CNS. *Eur J. Pharmacol.* **60**: 389–390 (1979).
10. Pasternak, G. W. Multiple opiate receptors: [^{3}H]ethylketocyclazocine receptor binding and analgesia. *Proc. Natl. Acad. Sci. U. S. A.* **77**:3691–3694 (1980).
11. Kosterlitz, H. W., F. R. S. Paterson, and S. J. Paterson. Characterization of opioid receptors in nervous tissue. *Proc. R. Soc. Lond. B Biol.* **210**:113–122 (1980).
12. Pfeiffer, A., and A. Herz. Evidence for a kappa-binding site in rat brain homogenate. *Neurosci. Lett. [Suppl. 7]* S184 (1981).
13. Pfeiffer, A., and A. Herz. Demonstration and distribution of an opiate binding site in rat brain with high affinity for ethylketocyclazocine and SKF 10047. *Biochem. Biophys. Res. Commun.* **101**:38–44 (1981).
14. Clayton, R. M., R. A. Shakespear, J. A. Duncan, and J. C. Marshall [with appendix by P. J. Munson and D. Rodbard]. Radioiodinated nondegradable gonadotropin releasing hormone analogues: new probes for the investigation of pituitary gonadotropin releasing hormone receptors. *Endocrinology* **105**: 1369–1381 (1979).
15. Hancock, A. A., A. L. DeLean, and R. J. Lefkowitz. Quantitative resolution of *beta*-adrenergic receptor subtypes by selective ligand binding. *Mol. Pharmacol.* **16**:1–9 (1979).
16. Munson, P. J., and D. Rodbard. Ligand: a versatile approach for characterization of ligand binding systems. *Anal. Biochem.* **107**:220–239 (1980).
17. Wuester, M., P. Rubini, and R. Schulz. The preference of putative proenkephalins for different types of opiate receptors. *Life Sci.* **29**:1219–1228 (1981).
18. Goldstein, A., S. Tachybania, L. I. Lowney, M. Hunkapiller, and L. Hood. Dynorphin-(1–13), an extraordinarily potent opioid peptide. *Proc. Natl. Acad. Sci. U. S. A.* **76**:6666–6670 (1979).
19. Zukin, R. S., and S. R. Zukin. Demonstration of [^{3}H]-Cyclazocine binding to multiple opiate receptor sites. *Mol. Pharmacol.* **20**:246–254, (1981).
20. Kosterlitz, H. A. W., S. J. Paterson, and L. E. Robson. Characterization of the kappa-subtype of the opiate receptor in the guinea pig brain. *Br. J. Pharmacol.* **73**:939–949 (1981).
21. Chang, K.-J., E. Hazum, and P. Cuatrecasas. Novel opiate binding sites selective for benzomorphan drugs *Proc. Natl. Acad. Sci. U. S. A.* **78**:4141–4145 (1981).
22. Ward, S. J., G. Metcalf, and J. M. H. Rees. Effects of proposed mu-, kappa- and sigma-agonists in rodents, in *Characteristics and Functions of Opioids.* Elsevier/North-Holland, Amsterdam, 497–498 (1978).
23. Michne, W. F., A. K. Pierson, and N. F. Albertson. Addendum. *Bull. Problems Drug Dependence* **36**:524–532 (1974).
24. Shearman, G. T., and A. Herz. The different discriminative stimulus properties of narcotics in the rat are the result of their interaction with different opiate receptors. *Neurosci. Lett. [Suppl. 7]* S222 (1981).
25. Shearman, G. T., and A. Herz. The different discriminative stimulus properties of narcotics in the rat are the result of their interaction with different opiate receptors. *J. Pharmacol. Exp. Ther.* In press (1982).
26. Pasternak, G. W., S. R. Childers, and S. H. Snyder. Opiate analgesia: evidence for mediation by a subpopulation of opiate receptors. *Science (Wash. D. C.)* **208**:514–516 (1980).
27. Law, P. L., H. H. Loh, and C. H. Li. Properties and localization of β-endorphin receptor in the rat brain. *Proc. Natl. Acad. Sci. U. S. A.* **76**:5455–5459 (1979).

Send reprint requests to: Dr. Albert Herz, Department of Neuropharmacology, Max Planck Institute for Psychiatry, Kraepelinstrasse 2, 8 Muenchen 40, Federal Republic of Germany.