MOLECULAR PHARMACOLOGY, 25:337-342

Site-Directed Alkylation of Multiple Opioid Receptors

I. Binding Selectivity[1]

IAIN F. JAMES AND AVRAM GOLDSTEIN

Addiction Research Foundation and Stanford University, Palo Alto, California 94304

Received October 4, 1983; Accepted January 19, 1984

SUMMARY

We report a method for measuring and expressing the binding selectivity of ligands for μ, δ, and κ opioid binding sites. We used radioligands that are partially selective for these sites in combination with membrane preparations enriched in each site. Enrichment was obtained by treatment of membranes with the alkylating agent β-chlornaltrexamine in the presence of appropriate protecting ligands, sufentanil for μ sites, [D-Ala^2, D-Leu^5] enkephalin for δ sites, and dynorphin A for κ sites. After enrichment for μ receptors, [^{3}H] dihydromorphine bound to a single type of site as judged by the slope of competition binding curves. After enrichment for δ or κ receptors, binding sites for [^{3}H][D-Ala^2,D-Leu^5]enkephalin and [^{3}H]ethylketocyclazocine, respectively, were still not homogeneous. There were residual μ sites in δ-enriched membranes but we found no evidence for residual μ or δ sites in κ-enriched membranes. We used this method to identify ligands that are highly selective for each of the three types of sites: Tyr-D-Ala-Gly-(Me)Phe-Gly-ol, sufentanil, and morphiceptin for μ sites; (D-Pen^2, D-Pen^5]enkephalin and [D-Pen^2,L-Pen^5]enkephalin for δ sites; and tifluadom and U50,488 for κ sites.

INTRODUCTION

Multiple types of opioid receptors were proposed on pharmacological grounds by Martin *et al.* (1) and later (with somewhat different typology) by Lord *et al.* (2). Evidence for multiple opioid receptors has also been obtained in numerous binding studies, such as that by Chang and Cuatrecasas (3) using radioligands chosen for their partial selectivities for one or another receptor type. There is evidence for five different types of opioid receptor, which are designated by the Greek letters μ, δ, κ, ϵ, and σ. Recent reviews summarize the rather extensive literature (4–6).

Ultimately, the similarities and differences among receptor types will be clarified by isolation and sequence analysis. At present, however, it would be useful to have a clear understanding of the functional differences among these receptor types, their relationships to endogenous ligands, and their physiological roles. These aims require methods of identifying and activating one receptor type without cross-reaction with other types; i.e., they require the development of agonists and antagonists that are highly selective for a single type of opioid receptor. Several of the compounds that have been used as "prototypical" ligands for different types of receptor are, in fact, poorly selective. For example, the "prototypical" κ ligand, EKC,[2] binds to both μ and κ receptors with high affinity (7), and the "prototypical" δ ligand, DADLE, binds almost equally well to δ and μ receptors (8). The need for highly selective ligands led us to consider how best to define and measure selectivity.

We distinguish between two kinds of selectivity, *binding selectivity* and *pharmacological selectivity*. Binding selectivity is the ratio of *affinities* for a given opioid ligand at the several types of binding sites. Pharmacological selectivity, on the other hand, is defined as the ratio of *potencies* at the several receptor types in a bioassay; it depends not only on affinity but also on the number of receptors in the tissue and the intrinsic activity (or efficacy) (9, 10) of the agonist. In this report, we deal only with binding selectivity; pharmacological selectivity is discussed in the accompanying paper (11).

We estimate affinities at the different types of opioid binding sites in the usual way, by competition between a

This work was supported by Grant DA-1199 from the National Institute on Drug Abuse.

[1] This is the first paper of a two-part series. See ref. 11 for the accompanying paper on Pharmacological Selectivity.

[2] The abbreviations used are: EKC, ethylketocyclazocine; DADLE, [D-Ala^2,D-Leu^5]enkephalin; β-CNA, β-chlornaltrexamine; DHM, dihydromorphine; RSI, receptor selectivity index; DAGO, Tyr-D-Ala-Gly-(Me)Phe-Gly-ol; DPLPE, [D-Pen^2,L-Pen^5]enkephalin; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; DTLET, [D-Thr^2,Leu^5]enkephalin-Thr^6; DSLET, [D-Ser^2,Leu^5]enkephalin-Ser^6; KRS, Krebs-Ringer solution.

0026-895X/84/030337-06$02.00/0

partially selective radioligand and a nonradioactive test ligand. We attempt to correct for the poor selectivity of the available radioligands by preparing membranes that are enriched in a single type of opioid binding site, so that effectively only that site is available for labeling. Modifying earlier approaches by Robson and Kosterlitz (12) and by Smith and Simon (13), we inactivate all but one receptor type by treating brain membranes with the site-directed alkylating agent, β-CNA (see ref 14), in the presence of protecting concentrations of a ligand that is as selective as possible for one type of site (15).

β-CNA itself, which is a naltrexone derivative, shows a modest preference for inactivating μ receptors, but this is of no importance to the method. When β-CNA is used in the presence of a high concentration of dynorphin A, high-affinity binding sites for [^{3}H]DHM and [^{3}H]DADLE are almost completely inactivated, but about 40% of the [^{3}H]EKC sites are preserved (15). Low-affinity components of [^{3}H]EKC binding are eliminated, and the affinity of [^{3}H]EKC for the remaining high-affinity sites is the same as observed initially. In treated membranes, the relative affinities of the various ligands, obtained by competition with [^{3}H]EKC, show that dynorphin A has protected κ sites selectively. We have not yet found protective ligands for μ and δ sites that are as selective as is dynorphin for κ sites. However, sufentanil and DADLE, respectively, protect μ and δ sites well enough to be useful in characterizing selectivity (15). With such preparations of brain membranes, each enriched in μ, δ, or κ receptors, we can obtain quantitative information about a wide variety of opioids. Essentially the same technique can be used in pharmacological tissue preparations to protect or inactivate a single receptor type selectively (16–18). An alternative approach, which has been applied successfully in binding assays but is not generally suitable for bioassays, is to use a radioligand that is partially selective for the preferred receptor type in the presence of high blocking concentrations of nonradioactive ligands that are partially selective for the nonpreferred types (19).

To describe the *binding selectivity profile* of a ligand we determine apparent K_i values at μ, δ, and κ binding sites, using the appropriate partially selective radioligands and membranes enriched in each type of site by treatment with β-CNA and appropriate protecting ligands. We express selectivity as the ratio, apparent K_i at a given site divided by apparent K_i at the highest-affinity site. We call this ratio the *receptor selectivity index*, RSI. We write these ratios in a standard order, μ, δ, κ (if relevant, one could add ε and σ). Instead of writing 1.0 for the highest-affinity site, we write the pK_i (negative logarithm to the base 10 of K_i, analogous to pH and pK_a in chemistry and Schild's pA_x in pharmacology) in brackets. For example, we found the selectivity profile for DADLE to be $2.1\mu - [8.83]\delta - 6500\kappa$. This means that DADLE has highest affinity for δ receptors [apparent K_i = antilog(−8.83) = 1.5 nM], is selective for δ over κ by more than 3 orders of magnitude, but is very poorly selective for δ over μ. This way of presenting the data emphasizes selectivity (which is our concern here) but contains all of the relevant quantitative information about a ligand.

Apparent K_i is calculated from IC_{50} in competition binding assays as described in the legend to Table 2. In some cases (where binding sites are still not homogeneous after β-CNA treatment and selective protection), apparent K_i is not a true estimate of the dissociation constant, hence the designation "apparent." However, since we are comparing competition with three different radioligands, used for technical reasons at different concentrations (and different fractions of K_d), it would not be correct to compare IC_{50} values directly. Use of apparent K_i values, when the population of binding sites is not homogeneous, results in underestimates of selectivity (see Discussion).

Here, we report binding selectivity profiles for a range of opioid ligands. We identify the following as highly selective ligands with RSI no less than about 100 at any nonpreferred site: DAGO, sufentanil, and morphiceptin for μ receptors; DPLPE and DPDPE for δ receptors; and tifluadom and U50,488 for κ receptors.

MATERIALS AND METHODS

Reagents. [^{3}H]DHM (65 Ci/mmole) and [^{3}H]DADLE (25 Ci/mmole) were obtained from Amersham (Arlington Heights, Ill.); [^{3}H]EKC (16 Ci/mmole) was from New England Nuclear Corporation (Boston, Mass.). Except where otherwise noted, all peptides were obtained from Peninsula Laboratories (San Carlos, Calif.). Normorphine hemihydrate was from Applied Science (Gardena, Calif.), naloxone hydrochloride from Endo Laboratories (Garden City, N. Y.), EKC methane sulfonate from Sterling Winthrop (Rensselaer, N. Y.), and sufentanil citrate from Janssen Pharmaceutical (New Brunswick, N. J.). The following ligands were generous gifts: Tyr-c[D-Lys-Gly-Phe] (20) from Dr. P. Schiller; DPLPE and DPDPE (21) from Dr. H. I. Mosberg; DTLET and DSLET (8, 22) from Dr. B. Roques; metkephamid ([D-Ala^2,(Me)Met^5]enkephalin amide) and its free acid (23) from Dr. R. C. A. Frederickson; ICI 154,129 (*N*,*N*-bisallyl-Tyr-Gly-Gly-ψ-(CH_2S)-Phe-Leu-OH) (24) from Dr. J. Holaday; U50,488 (25) from Dr. M. F. Piercey; DAGO (26), bremazocine (27), and tifluadom (28) from Dr. D. Romer. β-CNA was a generous gift from Drs. P. S. Portoghese and A. E. Takemori.

Preparation of membranes. Guinea pig brain membranes were prepared and treated with β-CNA and protecting ligands as described previously (15). A male Hartley guinea pig (300–500 g; Simonsen Laboratories, Gilroy, Calif.) was killed by decapitation. Brain tissue without cerebellum was homogenized using a Tissumizer (Tekmar, Cincinnati, Ohio) for 30 sec at 37° in 10 volumes of KRS of the following composition (millimolar): NaCl, 118; KCl, 4.75; $CaCl_2$, 2.54; KH_2PO_4, 1.19; $MgSO_4$, 1.20; $NaHCO_3$, 25; glucose, 11; choline chloride, 0.02; and mepyramine maleate (125 nM). The homogenate was centrifuged (14,500 × *g*, 37°, 30 min) and the pellet was resuspended in KRS, incubated for 20 min at 37°, cetrifuged a second time, and resuspended in KRS to a concentration of 16 mg of original tissue per milliliter. This suspension was incubated for 20 min with 10 nM β-CNA and protecting ligand (added 1 min before β-CNA). Membranes were then diluted at least 1:4 with KRS and centrifuged (here and below, 14,500 × *g*, 37°, 10 min), washed four times by resuspension in KRS, incubated for 20 min at 37°, and centrifuged again. Finally, membranes were washed twice in 50 mM Tris-HCl (pH 7.4) (or KRS if this buffer was to be used in the binding assay) by suspension and immediate centrifugation and then were resuspended in the binding assay buffer at room temperature to a concentration of 20 mg of original tissue per milliliter.

Radioreceptor binding assays. Binding of the opioid ligands [^{3}H]DHM, [^{3}H]DADLE, and [^{3}H]EKC was assayed as described elsewhere (15). The assay mixture contained 1 ml of membrane suspension

with radioligand and, where appropriate, competing ligand in a final volume of 2 ml of 50 mM Tris-HCl (pH 7.4) or KRS. Saturable binding was determined by competition with 10 μM naloxone. The membranes were incubated for 1 hr at room temperature, cooled on ice, filtered through Schleicher and Schuell No. 32 glass-fiber filters, washed three times in ice-cold assay buffer, and counted in 5 ml of Cytoscint. For experiments in KRS, all tubes were kept capped through the incubation until immediately before filtering in order to maintain the pH at 7.4. For saturation curves, binding was measured in triplicate at 15 different radioligand concentrations between 0.1 and 20 nM. Dissociation constants (K_d) and concentrations of binding sites (B_{max}) were estimated by nonlinear least-squares regression using the statistical analysis system program NLIN as described previously (15). For competition binding assays, the concentration of competing ligand required to reduce saturable binding of radioligand by 50% (IC_{50}) and the slope of the curve were estimated by linear regression from a Hill plot of log[L] against log(P/[100−P]), where [L] is concentration of competing ligand and P is percentage inhibition of saturable binding of radioligand. All data in tables and text have been rounded to two significant figures.

RESULTS

Estimates of parameters describing the binding of [^{3}H]DHM, [^{3}H]DADLE, and [^{3}H]EKC to untreated and treated guinea pig brain membranes are given in Table 1. We performed binding assays in the usual Tris buffer and in KRS. In most experiments, Scatchard plots for [^{3}H]DHM and [^{3}H]DADLE were linear, and regression analysis was consistent with a single site. Attempts to fit a two-site model either gave the same K_d for both sites or a ridiculously low K_d (e.g., 10^{-17} nM) for one of the sites. However, in three experiments with these ligands (of 22 over-all), the data could be fitted to a two-site model. Only the single-site results are reported in Table 1. For [^{3}H]EKC, details of binding curves have been reported elsewhere (15). As described previously, data for this ligand fitted a two-site model, but only parameters for the high-affinity site are considered here. Treatment of membranes with β-CNA in the presence of an appropriate protecting ligand reduced B_{max} for each of the radioligands, without affecting affinity. Changing buffer from Tris to KRS had little effect on binding affinities for any of the radioligands, but there was a change in B_{max} for [^{3}H]DHM and [^{3}H]DADLE.

TABLE 1

Parameters for binding of radioligands to untreated and treated membranes

Binding assays were performed as described under Materials and Methods. Parameters were estimated, for each experiment separately, by nonlinear regression on the binding isotherm, using the statistical analysis system program, NLIN. Data are means ± standard error of the mean for the number of independent experiments with different membrane preparations given under N. B_{max} is the site density per weight of original tissue. Treated membranes were reacted with 10 nM β-CNA in the presence of a protecting ligand, 100 nM sufentanil for [^{3}H]DHM sites, 1 μM DADLE for [^{3}H]DADLE sites, and 100 nM dynorphin A for [^{3}H]EKC sites, as described under Materials and Methods. Untreated membranes were incubated and washed exactly as treated membranes, but were not exposed to β-CNA or protector.

Radioligand	Buffer	N	Membrane preparation	K_d	B_{max}
				nM	*pmoles/g*
DHM	Tris	4	Untreated	2.4 ± 0.93	5.5 ± 0.66
DHM	Tris	4	Treated	2.2 ± 0.20	2.3 ± 0.64
DHM	KRS	3	Untreated	2.3 ± 0.42	1.0 ± 0.17
DADLE	Tris	4	Untreated	2.5 ± 0.24	7.5 ± 0.24
DADLE	Tris	4	Treated	2.6 ± 0.35	2.2 ± 0.24
DADLE	KRS	3	Untreated	6.9 ± 2.4	1.8 ± 0.30
EKC	Tris	4	Untreated	0.2 ± 0.10	2.6 ± 0.65
EKC	Tris	5	Treated	0.14 ± 0.04	1.3 ± 0.17
EKC	KRS	5	Untreated	0.36 ± 0.15	1.5 ± 0.86

Linear Scatchard plots and regression analysis compatible with a single-site model do not necessarily mean that a ligand is binding to only one type of site. For example, a theoretical Scatchard plot for binding of a single ligand to two types of site present in equal numbers but with 5-fold difference in affinity is a straight line with a correlation coefficient of 0.996 (29). Slopes of Hill plots for competition of sufentanil for [^{3}H]DHM and [^{3}H]DADLE binding sites on untreated membranes were 0.76 ± 0.06 and 0.50 ± 0.06 (means ± SEM, N = 7), respectively, suggesting that these radioligands do bind to more than one type of site.

We can use competition binding assays to follow the enrichment of each type of binding site in the β-CNA procedure by following changes in Hill slopes and estimates of IC_{50} when membranes are treated with various concentrations of β-CNA in the presence of a protecting ligand. For these experiments, we chose the most selective competing ligands that we had, so that Hill slopes would be sensitive to site heterogeneity. To follow enrichment of μ sites, we protected with sufentanil and measured the Hill slope and IC_{50} for competition between sufentanil and [^{3}H]DHM. To follow enrichment of κ sites, we protected with dynorphin A and measured competition between U50,488 (a highly selective κ ligand; see below) and [^{3}H]EKC. At the time these experiments were performed, we had not identified a highly selective δ ligand. We knew that [^{3}H]DADLE bound to both μ and δ receptors in untreated membranes (8). A selective μ ligand at low concentrations would compete with [^{3}H]DADLE for μ sites, and only at much higher concentrations would it compete for the δ sites. The slope of the competition curve should be shallow. On inactivation of μ receptors by β-CNA, with protection of δ receptors, the μ ligand would compete only at δ receptors. The IC_{50} should increase and the Hill slope should tend toward 1.0. We protected with DADLE and used the μ ligand sufentanil in this way to follow enrichment of δ receptors.

For all three radioligands, Hill slopes in these competition assays increased after treatment of membranes with β-CNA and protector (Fig. 1A). For [^{3}H]DADLE and [^{3}H]EKC, slopes did not reach unity. Hence, even after treatment, the population of binding sites for these ligands was heterogeneous. For [^{3}H]DADLE binding to δ-protected membranes, the source of heterogeneity is probably residual μ binding sites. When, in other experiments (data not shown), 60 nM DAGO was included in the incubation mixture to block binding to μ sites, the Hill slope for competiton between sufentanil and [^{3}H]DADLE increased from 0.86 ± 0.07 to 0.94 ± 0.07 (mean ± SEM, N = 3). From the slope in the absence of DAGO, it appears that we were able to inactivate more μ-sites

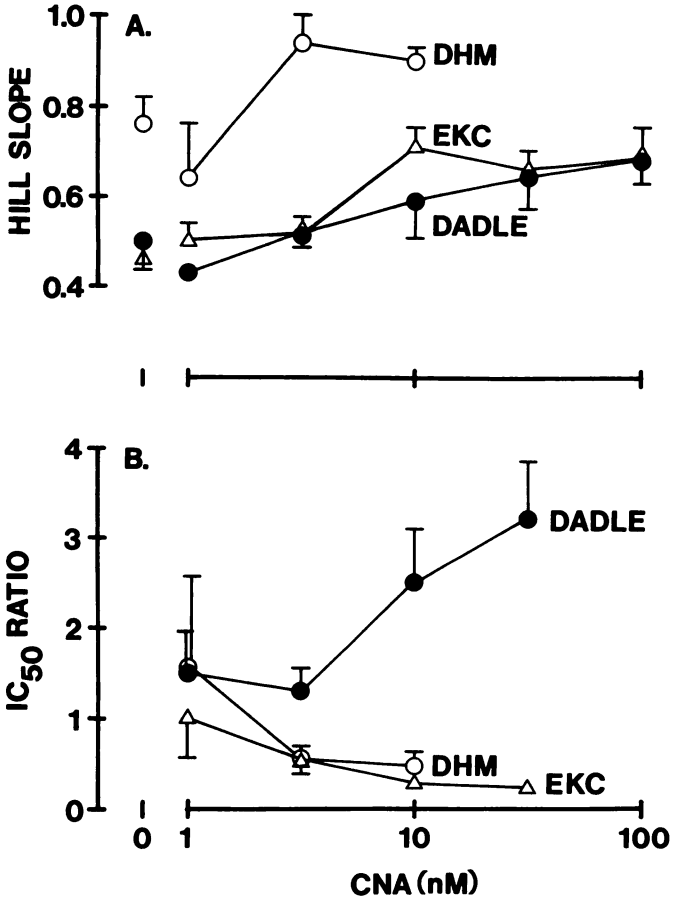

FIG. 1. *Effect of β-CNA with selective protection on slopes and IC_{50} values of competition curves*

Guinea pig brain membranes were treated with different concentrations of β-CNA in the presence of a protecting ligand, as described under Materials and Methods. Competition binding assays were performed in Tris buffer; slopes and IC_{50} values were estimated from Hill plots by linear regression. A, Effect of treatment on slopes of Hill plots; B, effect of treatment on IC_{50} values. Data are means ± standard error of the mean for three or four experiments on different membrane preparations. *IC_{50} ratio* is the ratio IC_{50} for treated membranes divided by IC_{50} for untreated membranes. For DHM (used at 1.3 nM) as radioligand, the protecting ligand was 100 nM sufentanil and the competer was sufentanil. For DADLE (1.8 nM), the protecting ligand was 1 μM DADLE and the computer was sufentanil. For EKC (0.6 nM), the protecting ligand was 100 nM dynorphin A and the competer was U50,488. Untreated membranes were incubated and washed in the same way as treated membranes but were not exposed to β-CNA or protector.

for these experiments than for the ones reported in Fig. 1. For κ-protected membranes, however, neither DAGO nor DPLPE (a δ receptor blocker) affected the Hill slope for competition between [^{3}H]EKC and U50,488. Slopes were 0.54 ± 0.05, 0.54 ± 0.06, and 0.53 ± 0.06 (mean ± SEM, N = 3) for incubations without blocking ligand, with 60 nM DAGO, and with 60 nM DPLPE, respectively.

As compared with untreated membranes, the effect of β-CNA treatment with selective protection was to increase the apparent affinity at each preferred site, while decreasing it at nonpreferred sites (Fig. 1B). Thus, ratios of IC_{50} after treatment to IC_{50} before treatment were less than 1.0 for competition of sufentanil with [^{3}H]DHM and of U50,488 with [^{3}H]EKC, in μ-protected and κ-protected membranes, respectively, and greater than 1.0 for competition of sufentanil with [^{3}H]DADLE in δ-protected membranes. This is to be expected when a radioligand binds at more than a single site in untreated membranes, and a competing ligand has some degree of selectivity for one of these sites. We call attention to an inaccurate statement of this point in one of our recent publications (ref. 15, p. 7573, line 27). The sentence should read: "When competing ligand is more selective for the major site than for the minor site, R is less than 1; and when it is less selective for the major site than for the minor site, R is greater than 1."

Table 2 presents binding selectivity profiles for a series of ligands supposedly selective for μ, δ, or κ sites. We did not include any endogenous opioid peptides, because those that we have tested were degraded rapidly in our assay system, and we have not yet been able to prevent this satisfactorily. There is evidence that the peptides included in Table 2 are stable under binding assay conditions (23, 31–33). Among the putative μ ligands, only DAGO, sufentanil, and morphiceptin showed satisfactorily high selectivity. Sufentanil, with highest affinity for

TABLE 2

Binding selectivity profiles for opioid ligands

Guinea pig brain membranes were exposed to 10 nM β-CNA in the presence of a protecting ligand, and estimates of IC_{50} were obtained in Tris buffer, as described under Materials and Methods. For [^{3}H]DHM (1.3 nM) as radioligand, membranes were prepared with 100 nM sufentanil as protecting ligand (μ sites). For [^{3}H]DADLE (1.8 nM), protecting ligand was 1 μM DADLE (δ sites). For [^{3}H]EKC (0.6 nM), protecting ligand was 100 nM dynorphin A (κ sites). Apparent dissociation constants (K_i, see text) were calculated from the equation $K_i = IC_{50}/[1 + (L/K_d)]$ (30), where K_d is the dissociation constant for the radioligand and L is its concentration. Binding selectivity profiles were calculated as described in the text. Data are means for three experiments on different membrane preparations, with binding assayed in tripicate in each experiment.

Competing ligand	Binding selectivity profile		
	μ	δ	κ
Putative μ ligands			
DAGO	[8.36]	130	170
Morphiceptin	[6.97]	270	86
Sufentanil	[10.3]	70	220
Tyr-c[D-Lys-Gly-Phe]	[9.08]	20	46
Normorphine	[8.15]	56	18
Naloxone	[9.14]	18	4.0
Putative δ ligands			
DPLPE	670	[8.52]	>600
DPDPE	780	[8.38]	>430
DSLET	20	[8.74]	>1,000
DTLET	7.8	[8.80]	1,600
Metkephamid, free acid	5.6	[9.21]	6,300
Metkephamid	[9.37]	2.6	300
ICI 154,129	9.3	[5.82]	5.8
DADLE	2.1	[8.82]	6,400
Putative κ ligands			
U50,488	1,300	12,000	[9.14]
Tifluadom	97	1400	[10.1]
Dynorphin A-(1–13) amide	30	78	[10.7]
EKC	7.5	78	[9.96]
Bremazocine	3.1	19	[10.1]

μ, was no more μ-selective than morphiceptin, which had 2,000-fold lower affinity; this exemplifies the independence of affinity and selectivity. The selectivity pattern, which has implications for molecular structure-activity relationships, was rather different for these two ligands, sufentanil rejecting κ three times more strongly than δ, a relationship exactly reversed with morphiceptin. The enkephalin analogue Tyr-c[D-Lys-Gly-Phe] was about as selective as normorphine, with approximately 20-fold discrimination between μ and the next-preferred site. Naloxone did not distinguish very well between μ and κ, but the rank order of affinities of the three types of receptor was the same as that found in bioassays (18).

Among the putative δ ligands, DPDPE and DPLPE were very selective. DSLET had very low affinity for κ sites, but its affinity was only 20-fold greater for δ than for μ. DTLET also did not bind well to κ sites but was slightly less selective than DSLET for δ as compared with μ. Zajac *et al.* (8) found RSI values for DSLET and DTLET in the same range as we report here, but found that DTLET was slightly more δ-selective than DSLET. Metkephamid showed a slight preference for μ sites, but, interestingly, the free acid was slightly δ-selective. The putative δ antagonist, ICI 154,129 showed only very weak δ selectivity. DADLE was evidently useless for discriminating μ from δ, although it rejected κ very strongly. We believe this serious deficiency of DADLE as a δ-selective ligand calls into question the large amount of published data in which [^{3}H]DADLE was used as a δ-selective radioligand.

Finally, among the putative κ ligands, tifluadom, and even more strikingly U50,488, combined exceptionally good selectivity with high affinity. Since these compounds are not peptides and therefore have the advantage of stability, they should prove very useful as radioligands for selective labeling κ receptors. It is not surprising to find that dynorphin A-(1–13) amide had both high affinity and good selectivity. EKC and bremazocine both proved to have rather poor κ:μ selectivity, as found elsewhere (19).

DISCUSSION

We have developed a method for measuring binding selectivity of ligands for multiple opioid receptors. We used radioligands that are partially selective for each receptor type in combination with brain membranes enriched in a particular type. After enrichment for δ or κ receptors, binding sites of [^{3}H]DADLE and [^{3}H]EKC, respectively, were still heterogeneous. There were residual μ receptors in the δ-enriched membranes, which accounted for the heterogeneity of [^{3}H]DADLE binding sites. In the κ-enriched membranes, since highly selective blockers of μ and δ sites had no effect on the Hill slope, we concluded that there were no residual μ or δ sites. The heterogeneity of [^{3}H]EKC binding sites could be caused by subtypes of κ receptors. Other groups have found evidence for κ-receptor subtypes (34, 35). The effect of heterogeneity of both [^{3}H]DADLE and [^{3}H]EKC binding will be to overestimate the apparent K_i for competing ligands that have the same receptor preference as the radioligand and to underestimate apparent K_i for those that have preferences different from the radioligand. This means that RSI values will be underestimated. The profiles presented in Table 2, therefore, are lower limits of selectivity. In spite of these sources of error, measurement of selectivity using the method described here should offer a considerable improvement over the use of partially selective ligands alone. For example, the "apparent selectivity profile" for dynorphin A-(1–13) amide with untreated membranes was "6.9μ − 10δ − [10.2] κ," in good agreement with our very first measurements of dynorphin binding (17). With treated membranes, the selectivity profile was 30μ − 78δ − [10.7] κ, considerably different from the previous result and in better agreement with what we know from pharmacological assays (16, 17).

One important use of selectivity profiles is the identification of highly selective ligands that could be made radioactive and become true prototypical radioligands for the different types of binding sites. The necessary degree of selectivity of a radioligand for its preferred site is often not appreciated, but it can be calculated from the mass-law equations. Suppose there are an equal number of preferred, high-affinity (A) sites, and nonpreferred, lower-affinity (B) sites. If the radioligand is used at a concentration equal to its K_d at A sites, and if its affinity for B sites is 10-fold less than for A sites, fully 15% of the specifically bound radioactivity will be at B sites. If used at a concentration of only 0.5 K_d, 13% of bound radioligand will still be at B sites. Matters become significantly worse if there are more low-affinity sites than high-affinity sites, a frequent occurrence. Even with a modest 2-fold excess of B sites over A sites, the 15% computed above becomes 26%. The essential point is that, unless a radioligand is selective for its preferred receptor type by at least 100-fold over any other type, its use may lead to erroneous interpretations. From our results, radioligands that meet (or nearly meet) this criterion and have sufficiently high affinity would be DAGO (or sufentanil) for μ sites, DPLPE or DPDPE for δ sites, and U50,488 (or tifluadom) for κ sites. DAGO (19) and sufentanil (36) have already been used as radioligands.

Our measurements of selectivity were made on guinea pig brain membranes in Tris buffer at 23°; it would be incorrect to extrapolate our results to other conditions. We attempted to make the selectivity profiles more general (and especially more relevant to physiological conditions) by repeating the binding experiments in KRS. However, we encountered technical difficulties that prevented this. The reduction in B_{max} for [^{3}H]DHM and [^{3}H]DADLE in KRS as compared with Tris buffer (Table 1) means that the fraction of total ligand binding represented by saturable binding was reduced considerably, as noted before in this laboratory (33). Inactivation of even more binding sites during the treatment with β-CNA reduced saturable binding to less than 10% of total binding when radioligands were used at concentrations close to their K_d. Clearly, it would be impossible to obtain reliable results under these conditions. Measurement of selectivity profiles in KRS must await synthesis of three highly selective radioligands; under these circumstances

there would be no need to inactivate any binding sites with β-CNA.

Those agonists that have been tested in pharmacological assays showed qualitatively the same selectivity as we found in our binding assays. Thus, sufentanil and DAGO are selective for μ receptors in mouse vas deferens (37), DPDPE and DPLPE are selective for δ receptors in the same tissue (21), and dynorphin peptides are selective for κ receptors in guinea pig ileum and mouse vas deferens (17, 38).

Comparison of highly selective ligands for each type of opioid binding site can provide insight into the distinctive properties of each site in relation to the structural features of its preferred ligand. Selectivity at a preferred site can obviously arise from an "optimal fit" associated with high affinity, and comparisons of molecular models of the more rigid selective ligands may give clues to the requirements for that fit. Selectivity can also arise from preferential rejection of nonpreferred sites (e.g., by steric hindrance), without unusually high affinity for the preferred site, as with morphiceptin.

ACKNOWLEDGMENTS

We thank Denise Hirai, Asha Naidu, and Madeline Rado for assistance. We are grateful to our colleagues, already cited in the text and tables, who generously furnished opioid ligands for testing. We are expecially indebted to Drs. P. S. Portoghese and A. E. Takemori, who generously made β-CNA available for this investigation.

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Send reprint requests to: Dr. Avram Goldstein, Addiction Research Foundation, 701 Welch Road, Palo Alto, Calif. 94304.