MOLECULAR PHARMACOLOGY, 25:38–45

Adrenal Medullary Opiate Receptors

Pharmacological Characterization in Bovine Adrenal Medulla and a Human Pheochromocytoma

E. CASTANAS,[1] P. GIRAUD,[1] Y. AUDIGIER,[2] R. DRISSI,[1] F. BOUDOURESQUE,[1] B. CONTE-DEVOLX,[1] AND C. OLIVER[1]

Laboratoire de Médecine Expérimentale, ERA 982 Centre National de la Recherche Scientifique, UER Nord, 13326 Marseille Cedex 15, France, et Laboratoire de Pharmacologie et de Toxicologie Fondamentale du Centre National de la Recherche Scientifique, 31400 Toulouse, France

Received January 24, 1983; Accepted August 24, 1983

SUMMARY

We have characterized the opiate binding sites on the membranes of bovine adrenal medulla and human pheochromocytoma, using ^{3}H-labeled D-Ala^2-D-Leu^5-enkephalin ([^{3}H]DADLE), [^{3}H]etorphine, and [^{3}H]ethylketocyclazocine ([^{3}H]EKC). Binding was stereoselective in both membrane preparations. Association and dissociation kinetics showed that steady state was achieved after 20–25 min of incubation at 37°. Saturation experiments were performed in the absence or in the presence of morphiceptin (1 μM), which masks the *mu* sites, D-Ser^2-Leu-enkephalin-Thr^6 (100 nM), which masks *delta* sites, or DADLE (5 μM), which was found to mask the *delta*, *mu*, and benzomorphan receptor. Taking into consideration the affinities of the three radioligands used (DADLE identifying the *delta* and *mu* sites when used in the nanomolar range; etorphine identifying the *delta*, *mu*, and benzomorphan sites; EKC identifying the *delta*, *mu*, *kappa*, and benzomorphan receptors) we have characterized pharmacologically the opiate sites present on bovine and human membranes. Human pheochromocytoma membranes contained (a) *mu* binding sites (15 fmoles/mg of protein, K_D [^{3}H]etorphine 1.0 nM, [^{3}H]EKC 5.4 nM, [^{3}H]DADLE 5.6 nM); (b) *kappa* sites (41 fmoles/mg of protein, K_D [^{3}H]EKC 1.0 nM); (c) benzomorphan sites (115 fmoles/mg of protein, K_D [^{3}H]etorphine and [^{3}H]EKC 1.0 nM). On bovine membranes we have detected (a) *delta* binding sites (10 fmoles/mg of protein, K_D [^{3}H]DADLE 0.7 nM); (b) *mu* sites (24 fmoles/mg of protein, K_D [^{3}H]DADLE 2.9 nM, [^{3}H]etorphine 0.2 nM, [^{3}H]EKC 3.4 nM); (c) *kappa* sites (12 fmoles/mg of protein, K_D [^{3}H]EKC 0.4 nM); (d) benzomorphan sites (80 fmoles/mg of protein, K_D [^{3}H]etorphine 0.2 nM, [^{3}H]EKC 1.3 nM); (e) a residual high-affinity (20 fmoles/mg of protein, K_D 0.2 nM) site identified by [^{3}H]etorphine in the presence of 5 μM DADLE. The relative proportions of benzomorphan sites were equal in both tissues (65% of the high-affinity sites) whereas *kappa* receptors were more abundant on human membranes (25%) than on bovine membranes (9% of the high-affinity sites).

INTRODUCTION

The adrenal medulla of different species contains and secretes considerable amounts of opioid peptides. Enkephalins (M-Enk[3] and L-Enk) and their precursors as well as dynorphin(s) share the greater intraglandular concentrations (see ref. 1 for a review). Although the role of endogenous opioids at the adrenomedullary site is obscure, evidence exists that, in both normal and neoplastic tissues, these agents could represent modulators of chromaffin cell secretion (1–3) via opioid receptors present on the membrane of chromaffin cells.

Pharmacological studies on animal chromaffin cells and human pheochromocytomas in primary culture have shown a reversible stereospecific action of opiates on cell

This work was supported in part by grants from the Institut National de la Santé et de la Recherche Médicale (CRL 81 40 15), Centre National de la Recherche Scientifique, Fondation pour la Recherche Médicale, and Faculté de Médecine de Marseille.

[1] Laboratoire de Médecine Experimentale, ERA 982 CNRS, UER Nord, 13326 Marseille Cedex 15, France.

[2] Laboratoire de Pharmacologie et de Toxicologie Fondamentale du CNRS, 31400 Toulouse, France.

[3] The abbreviations used are: Enk, enkephalin; EKC, ethylketocyclazocine; DADLE, D-Ala^2-D-Leu^5-enkephalin; DSLET, D-Ser^2-Leu-enkephalin-Thr^6.

0026-895X/84/010038-08$02.00/0

secretion, suggesting the presence of opiate receptors (1–3). Chavkin *et al.* (4) and Saiani and Guidotti (5) have reported stereospecific opiate sites on membranes of bovine adrenal medullas.

In the present study, opiate sites present on membranes of bovine adrenal medullas and a human pheochromocytoma were characterized pharmacologically, and receptor distributions on these two kinds of tissues were compared.

MATERIALS AND METHODS

Tumors were obtained in the operating room, immediately frozen in liquid nitrogen, and stored at −80° until use.

Bovine adrenals were obtained from a local slaughterhouse and kept on ice. The medulla was separated from the cortex within 1 hr after the animals were killed.

Membranes were prepared as follows: pheochromocytomas (progressively thawed at 0°) or bovine adrenal medullas were suspended in 10 volumes of 50 mM Tris-HCl buffer (pH 7.4) (assay buffer) and homogenized successively with an Ultra-Turax (30-sec medium speed) and a glass-Teflon homogeneizer (1500 rpm, five strokes). Crude membrane fractions were prepared by differential centrifugation between 1,500 × *g* (15 min) and 30,000 × *g* (20 min), and washed once in 10 volumes of assay buffer. In order to dissociate any endogenously bound peptide, membranes were preincubated at 37° for 30 min. Two additional washes were made in 10 volumes of assay buffer. Finally, membranes were suspended in assay buffer to give a concentration of 1–2 mg of protein per milliliter.

Binding experiments were performed in a final volume of 1.0 ml (0.5-ml membrane suspension, 0.1 ml of radioligand and 0.4 ml of assay buffer containing the different effectors) at 37° for 30 min. Bound radioactivity was separated with filtration through Whatman GF/B filters; filters were washed with 15 ml of ice-cold assay buffer and counted.

[^{3}H]EKC (specific activity 15 Ci/mmole) and [^{3}H]DADLE (specific activity 45 Ci/mmole) were obtained from New England Nuclear Corporation. [^{3}H]Etorphine (specific activity 40 Ci/mmole) was from Amersham. Morphiceptin and DADLE were purchased from Sigma Chemical Company. Dextrorphan and levorphanol were gifts from Hoffmann-La Roche. EKC, ketocyclazocine, and cyclazocine were gifts from Sterling-Winthrop. Morphine was from Francopia, fentanyl from Jansen, and naloxone from Endo Laboratories. DSLET was a gift from Pr. B. Roques, Paris.

Data analysis was performed by linear regression methods from Scatchard plots.

RESULTS

Association and Dissociation Kinetics

Kinetics of association and dissociation of [^{3}H]DADLE, [^{3}H]etorphine, and [^{3}H]EKC on human pheochromocytoma membranes are presented in Fig. 1. Kinetics on bovine adrenal medullary preparations presented a similar pattern. Maximal binding was achieved for all three ligands after 20 min of incubation. Dissociation in the presence of an excess of levorphanol (50 μM) reached a plateau after 25 min of incubation. The results of association and dissociation kinetics studies confirmed that, in our experimental conditions (30-min incubation), steady state was achieved in all cases.

Stereoselectivity

Table 1 shows the competition for [^{3}H]DADLE, [^{3}H]etorphine, and [^{3}H]EKC binding to bovine and human membranes by levorphanol (10 μM) and dextrorphan (10 μM). Minor competition was obtained by dextrorphan as compared with levorphanol, confirming the stereoselectivity of opiate binding on membranes. A concentration of 10 μM levorphanol was used throughout this study for the determination of nonspecific binding.

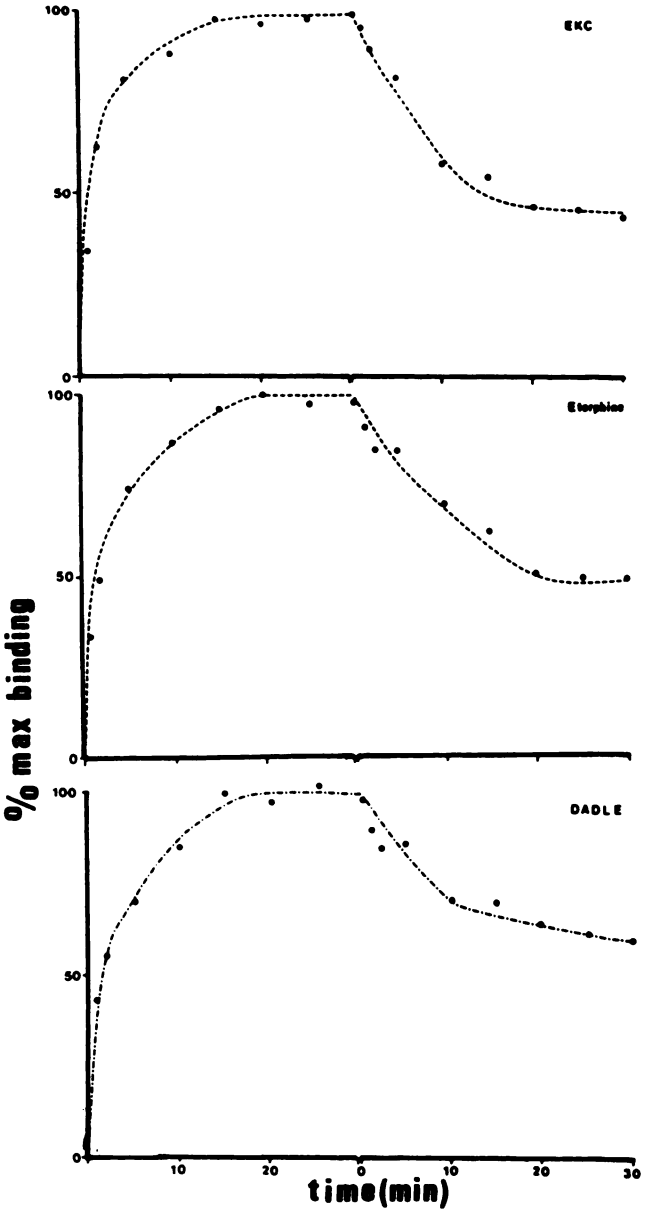

FIG. 1. *Kinetics of association and dissociation of radiolabeled opioids to human pheochromocytoma membranes*

Membranes were prepared as described under Materials and Methods. Total binding is presented. [^{3}H]EKC (0.91 nM), [^{3}H]etorphine (0.84 nM), and [^{3}H]DADLE (5.20 nM) were incubated with human membranes (0.6 mg/ml). At 1, 2, 5, 10, 15, 20, 25, and 30 min, aliquots were withdrawn, rapidly filtered on Whatman GF/B filters, washed with 15 ml of ice-cold assay buffer, and counted. After the end of the association period, membranes were washed with cold assay buffer and reincubated in the presence of 50 μM levorphanol. At the previously indicated times, aliquots were taken and bound radioactivity was separated and counted. After 30 min of association, [^{3}H]EKC binding was 0.7% of the total, [^{3}H]etorphine binding was 0.9% of the total, and [^{3}H]DADLE binding was 0.07% of the total.

TABLE 1

Stereoselectivity of opiate binding to adrenal medullary membranes

Membranes were prepared as described under Materials and Methods. Opiate binding was performed at 37° for 30 min in the absence (B_0) or the presence of 10 μM levorphanol or dextrorphan. Total binding is presented. Results are means ± standard error of the mean (n = 3). Protein concentration was 1.0 mg protein/ml in both cases (mean ± standard error of the mean of triplicate determinations in a single experiment).

	Ligand					
	[^{3}H]EKC (270,932 ± 1,150 total cpm)		[^{3}H]etorphine (312,955 ± 3,123 total cpm)		[^{3}H]DADLE (315,723 ± 2,320 total cpm)	
	Human	Bovine	Human	Bovine	Human	Bovine
	cpm bound					
B_0	3,317 ± 138	4,468 ± 203	5,611 ± 213	4,468 ± 205	2,577 ± 138	3,052 ± 98
Levorphanol	1,702 ± 79	2,391 ± 26	3,613 ± 132	2,391 ± 26	1,978 ± 132	1,053 ± 37
Dextrorphan	2,938 ± 103	4,212 ± 107	4,832 ± 217	4,212 ± 107	2,337 ± 201	2,838 ± 87

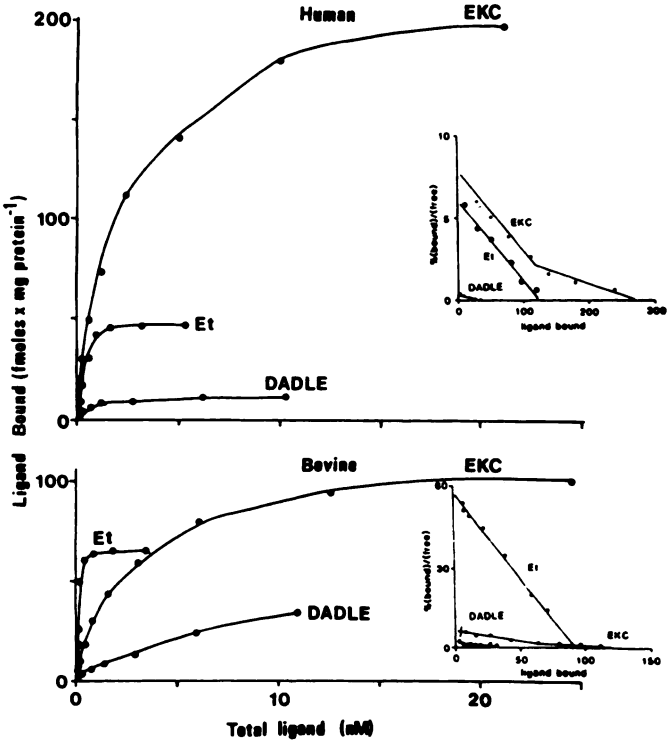

FIG. 2. *Saturation curves of [^{3}H]EKC, [^{3}H]etorphine, and [^{3}H]DADLE to human and bovine membranes*

Specific binding is presented. Binding experiments were performed at 37° for 30 min, using the indicated concentrations of the three radioligands. Nonspecific binding was determined in the presence of 10 μM levorphanol. Protein concentration was 0.60 mg/ml for human membranes and 1.0 mg/ml for bovine membranes. Incubation volume: 1 ml. *Inset*: Scatchard plots of the data. The abcissa is expressed as femtomoles bound per milligram of protein. Linear regression methods were used for the estimation of B_{max} and K_D.

Stability of Radiolabeled Opiates

The stability of radiolabeled DADLE, EKC, and etorphin was tested using thin-layer chromatography as described by the supplier (silica gel plates Whatman LHP-KF). The systems used were as follows: [^{3}H]DADLE, 1-butanol:acetic acid:water, 4:1:1; [^{3}H]etorphine, ethanol:acetic acid:water, 6:3:1; [^{3}H]EKC, chloroform:methanol, 9:1. No degradation was observed after 30 min of incubation for either radioligand.

Saturation Curves

On both bovine and human membranes, [^{3}H]DADLE, [^{3}H]EKC, and [^{3}H]etorphine showed concentration-dependent and saturable specific binding (Fig. 2). Scatchard analysis (Fig. 2, *inset*) showed an apparent homogeneity of binding sites for [^{3}H]DADLE and [^{3}H]etorphine, whereas curvilinear plots were obtained using [^{3}H]EKC (see also Tables 2 and 3). Comparison of human and bovine membranes showed a greater number of binding sites identified by [^{3}H]EKC on human membranes, whereas [^{3}H]DADLE and [^{3}H]etorphine identified a greater number of sites on bovine than on human membranes.

DADLE Competition for Tritiated Ligands

Attali *et al.* (6) have recently reported that in the lumbosacral region of guinea pig spinal cord, possessing

TABLE 2

Binding parameters of a human pheochromocytoma membrane preparation

Binding experiments were performed as described under Materials and Methods. Modification of binding parameters of [^{3}H]EKC, [^{3}H]etorphine, and [^{3}H]DADLE upon the addition of 5 μM DADLE, 1 μM morphiceptin, or 0.1 μM DSLET. Two apparent binding sites were found for [^{3}H]EKC binding but only one apparent site was found when [^{3}H]etorphine or [^{3}H]DADLE was the labeled ligand (see also Fig. 2). Results are expressed as means ± standard error of the mean of three separate experiments where the intraassay coefficient of variation was <10% (2 SD).

Ligand	High-affinity sites		Low-affinity sites	
	B_{max}	K_D	B_{max}	K_D
	fmoles/mg protein	*nM*	*fmoles/mg protein*	*nM*
[^{3}H]EKC				
No addition	163 ± 4	0.94 ± 0.03	275 ± 15	4.15 ± 0.13
DADLE	41 ± 2	0.98 ± 0.02	114 ± 8	6.72 ± 0.11
Morphiceptin	159 ± 5	0.95 ± 0.05	258 ± 4	6.70 ± 0.10
DSLET	157 ± 4	1.0 ± 0.03	282 ± 12	6.83 ± 0.12
[^{3}H]Etorphine				
No addition	110 ± 2	0.94 ± 0.05	—	—
DADLE	Loss of specific binding		—	—
Morphiceptin	101 ± 3	0.95 ± 0.04	—	—
DSLET	99 ± 7	1.04 ± 0.05	—	—
[^{3}H]DADLE				
No addition	—	—	18 ± 3	5.60 ± 0.03
Morphiceptin	—	—	Loss of specific binding	
DSLET	—	—	19 ± 2	5.35 ± 0.05

TABLE 3

Binding characteristics of bovine adrenomedullary membranes

Modification of binding characteristics upon the addition of 5 μM DADLE, 1 μM morphiceptin, or 0.1 μM DSLET. Binding experiments were performed as described under Materials and Methods. Results are expressed as the mean of four separate experiments ± standard error of the mean. Interassay coefficient of variation ≤10% (2 SD).

Ligand	High-affinity sites		Low-affinity sites	
	B_{max}	K_D	B_{max}	K_D
	fmoles/mg protein	*nM*	*fmoles/mg protein*	*nM*
[^{3}H]EKC				
No addition	114 ± 5	1.38 ± 0.04	165 ± 3	3.06 ± 0.05
DADLE	12 ± 1	0.41 ± 0.02	97 ± 5	18.50 ± 1.00
Morphiceptin	116 ± 4	1.37 ± 0.02	138 ± 5	3.80 ± 0.04
DSLET	93 ± 5	1.43 ± 0.03	173 ± 8	21.2 ± 0.70
[^{3}H]Etorphine				
No addition	125 ± 3	0.16 ± 0.01	—	—
DADLE	20 ± 2	0.19 ± 0.01	—	—
Morphiceptin	99 ± 3	0.24 ± 0.02	—	—
DSLET	94 ± 5	0.25		
[^{3}H]DADLE				
No addition	30 ± 3	2.20 ± 0.06	—	—
Morphiceptin	10 ± 2	0.71 ± 0.02	—	—
DSLET	17 ± 3	2.97 ± 0.03	—	—

only *kappa* sites, DADLE competition curves for [^{3}H]EKC and [^{3}H]etorphine binding were different: etorphine specific binding was totally competed for by DADLE, whereas residual 40% binding was observed in the case of [^{3}H]EKC. They have proposed the subdivision of the *kappa* receptors into DADLE-sensitive sites, which represent the benzomorphan sites, and DADLE-insensitive sites (former *kappa* receptors). In order to characterize *kappa* and benzomorphan sites further, it would be of interest to determine whether such a phenomenon also occurs on bovine and human adrenomedullary membranes.

Competition curves for [^{3}H]EKC and [^{3}H]etorphine binding by DADLE are presented in Fig. 3. In both cases, curves were multiphasic. This is attributed to multiple opiate receptor populations, presenting different affinities for DADLE (see later results and Discussion). One can observe that (a) [^{3}H]Etorphine was totally competed for by DADLE in human membranes. A biphasic curve was found with $IC_{50}1$ of 1 nM and $IC_{50}2$ of about 100 nM. In contrast, in bovine membranes, a residual specific binding was detectable even in the presence of 10 μM DADLE. (b) [^{3}H]EKC in both types of membranes showed residual binding in the presence of DADLE. This binding represented 50% of the total binding on human membranes and 35% of the total binding in the case of bovine membranes. As in the case of competition for [^{3}H]etorphine, curves were biphasic.

Competition for [^{3}H]DADLE by DADLE (data not shown) was biphasic in the case of bovine membranes (IC_{50} of the two components in the range of 0.1 nM and 10 nM), whereas monophasic curves with an IC_{50} of about 1 nM were observed in human pheochromocytoma membranes.

Effect of Different Effectors on Saturation Binding

The three radioligands used are not specific for one class of binding sites. DADLE (at concentrations ≤10 nM) can bind to *delta* and *mu* subtypes; etorphine can identify the *delta*, *mu*, and benzomorphan sites; and EKC can bind to the *delta*, *mu*, benzomorphan, and *kappa* sites (7–9). In order to discriminate between these binding sites, effectors which mask one or more types of sites must be used during saturation experiments. Effectors used in the present study were morphiceptin (1 μM), considered as a pure *mu* agonist (10); DSLET (100 nM), a pure *delta* agonist (11); and DADLE (5 μM), which, as indicated above, can mask the *delta*, *mu*, and benzomorphan sites (7). The results of these experiments are presented in Tables 2 and 3.

[^{3}H]DADLE binding. Morphiceptin addition during

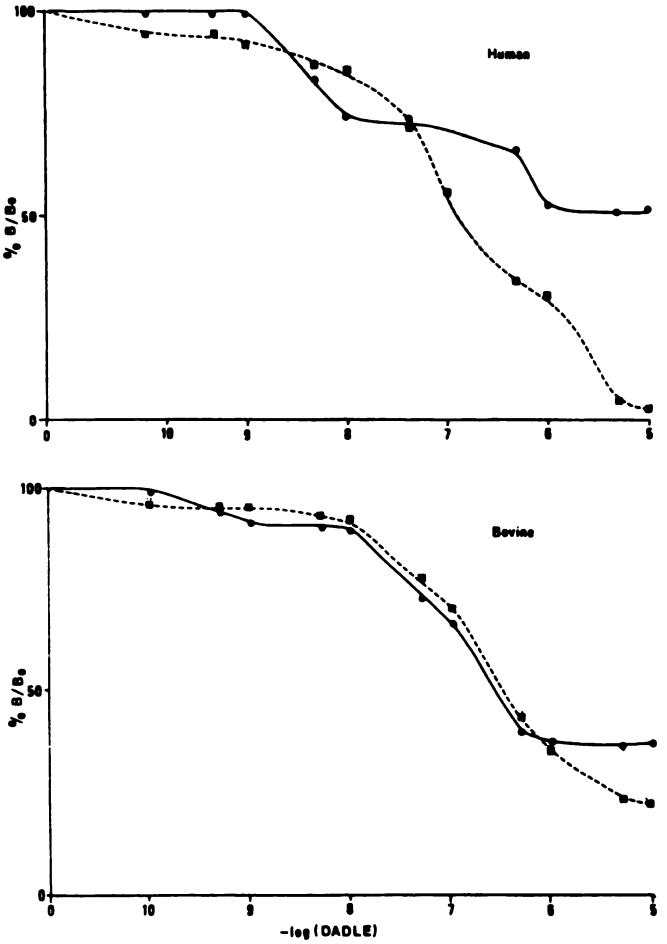

FIG. 3. *DADLE competition for [^{3}H]EKC (●——●) and [^{3}H]etorphine (■- -■)*

Human (0.5 mg/ml) and bovine (0.7 mg/ml) membranes were incubated for 30 min at 37° with 1.0 nM [^{3}H]EKC or 0.36 nM [^{3}H]etorphine in the presence of the indicated concentrations of unlabeled DADLE. Bound radioactivity was separated by filtration. Maximal binding was 2.2% and 5.4% of the total radioligand added in the case of human membranes and 4.0% and 16.9% in the case of bovine membranes using [^{3}H]EKC and [^{3}H]etorphine, respectively. Nonspecific binding determined in the presence of 10 μM levorphanol was 36% ([^{3}H]EKC) and 30% ([^{3}H]etorphine) of the total radioligand bound on human and 30% ([^{3}H]EKC) and 16.5% ([^{3}H]etorphine) on bovine membranes. Nonspecific binding was systematically subtracted from each point, which was determined in triplicate with a coefficient of variation not exceeding 10% (2 SD).

saturation curves abolished specific binding in the case of human membranes, and it diminished drastically the binding on bovine membranes, indicating that DADLE identifies only *mu* sites on human tissue. This fact was supported by the rather high K_D of DADLE for its receptors on human preparations, comparable to the K_D reported by other authors for DADLE binding on *mu* sites (7, 9), and by the fact that DSLET addition, which masks *delta* sites, did not provoke any change in DADLE binding parameters. The residual DADLE binding in the presence of morphiceptin in the case of bovine membranes can be attributed to *delta* sites. This was confirmed by the equivalent diminution of binding sites in the presence of 100 nM DSLET.

[^{3}H]Etorphine binding. As discussed above, etorphine can bind to the *delta*, *mu*, and benzomorphan receptors. Morphiceptin addition during saturation experiments provoked a decrease in the number of binding sites with no change in the apparent affinity of etorphine for the remaining receptors. The number and the apparent K_D of *mu* sites for etorphine can therefore be calculated. DSLET addition resulted in no change in binding characteristics in human membranes, whereas a 29 fmoles/mg of protein diminution of sites with no change in the apparent affinity of etorphin was noted in bovine membranes. This diminution has been attributed to *delta* sites. DADLE addition during saturation experiments abolished specific binding in the case of human membranes, and drastically diminished it in the case of bovine tissue. No change in the apparent affinity of etorphine was noted. The existence of a residual component of etorphine binding not masked by DADLE was in accord with the results of competition curves (Fig. 3).

[^{3}H]EKC binding. As discussed earlier, EKC is able to identify all four types of binding sites. Morphiceptin addition did not interfere with the high-affinity component of [^{3}H]EKC binding while it diminished the low-affinity component in both types of membranes. This decrease (17 fmoles/mg of protein in human membranes and 26 fmoles/mg protein in bovine membranes) corresponded well with the number of sites masked by morphiceptin during [^{3}H]DADLE (18 and 21 fmoles/mg of protein for human and bovine membranes, respectively) and [^{3}H]etorphine binding (10 and 25 fmoles/mg of protein, respectively). DSLET addition provoked no change in binding characteristics of [^{3}H]EKC on human membranes, whereas a 21 fmoles/mg of protein diminution of the high-affinity component of [^{3}H]EKC binding was found in bovine membranes, with no change of the apparent K_D of [^{3}H]EKC for the remaining sites. The observed decrease in binding was comparable with the one found during [^{3}H]DADLE (10 fmoles/mg of protein) and [^{3}H]etorphine binding (29 fmoles/mg of protein). DADLE addition (masking *delta*, *mu*, and benzomorphan receptors) showed the existence of DADLE-insensitive sites (*kappa* receptors) on both membranes. The decrease in binding by DADLE (122 fmoles and 111 fmoles/mg of protein for human and bovine tissues) corresponded to the number of sites masked by DADLE during [^{3}H] etorphine binding (110 and 105 fmoles/mg of protein, respectively).

Opiate Binding Site Characterization

Based on the results presented earlier in this study, the number of each type of opiate receptors could be calculated: the *mu* sites were estimated from saturation experiments (Tables 2 and 3) using morphiceptin. *Delta* sites were estimated as the residual [^{3}H]DADLE binding in the presence of morphiceptin or from saturation experiments using DSLET. Benzomorphan sites were calculated as the number of DADLE-sensitive sites minus the sum of *mu* and *delta* sites. *Kappa* receptors represented the residual EKC binding after the addition of DADLE. The K_D of each site was calculated either directly from Scatchard plots (for *delta* and *kappa* sites) or from the observed change in the apparent K_D of each ligand in the absence or in the presence of effectors. The results of these calculations are presented in Table 4.

Although etorphine is considered a ligand of the *delta*, mu, and benzomorphan receptors (7) and DADLE was found to abolish its specific binding (6), a residual site was found on bovine membranes in the presence of 5 μM DADLE in the present study (see Fig. 3 and Table 3). The possibility that this site might be a *kappa* receptor also identified by [^{3}H]etorphine was examined by competing for [^{3}H]etorphine binding by EKC in the absence and in the presence of 5 μM DADLE. No change in the apparent K_I of EKC was observed (K_I = 16.9 ± 0.6 nM in the absence and 15.0 ± 0.5 nM in the presence of DADLE; data not shown). The K_I observed during these competition studies indicated that this site must not represent a *kappa* receptor because of the great discrepancy between the dissociation (0.4 nM) and the inhibition (15 nM) constants observed. The K_I of this site for EKC was similar to the one found by Chang *et al.* (9) for the rat brain benzomorphan receptor (16 nM), but quite different from the K_I of EKC for the DADLE-insensitive site found by Attali *et al.* (6) in the guinea pig spinal cord (0.1 nM). The same authors reported a K_I for EKC competition for the DADLE-sensitive site (benzomorphan receptor) of 0.3 nM. These discrepancies suggest that it is unprobable that this site is a *kappa* receptor. It is equally unprobable that 5 μM DADLE would be unable to mask benzomorphan sites.

TABLE 4

Calculation of receptor types on bovine and human membranes

Receptor types were calculated as described in the text. ND, Not detectable.

Site type	K_D		B_{max}		Tritiated ligand used
	Bovine	Human	Bovine	Human	
	nM		*fmoles/mg protein*		
Delta	0.7	ND	10	ND	DADLE
	1.4	ND	21	ND	EKC
	0.1	ND	29	ND	Etorphine
Mu	3.4	5.4	26	17	EKC
	2.9	5.6	21	18	DADLE
	0.2	1.0	25	10	Etorphine
Kappa	0.4	1.0	12	41	EKC
Benzomorphan	1.3	1.0	72	118	EKC
	0.2	1.0	89	101	Etorphine
	0.2	—	20	—	Etorphine

Kappa Binding Sites on Pheochromocytoma Membranes

Human pheochromocytomas are heterogeneous tumors. A large heterogeneity in the content of catecholamines, endogenous opioids, and opioid precursors, as well as their secretory characteristics, has been reported (1–3). Unfortunately, they are also rare tumors, and most of the time small amounts of tissue are available.

In addition to the human tumor analyzed, we have studied three other tumors. The scarcity of the material did not permit us to completely characterize opioid receptors present on their membranes. An attempt to characterize *kappa* sites is presented in Table 5: a great heterogeneity of [^{3}H]EKC binding was found. Dissociation constants and site concentrations varied from 0.37 to 1.13 nM and from 30 to 163 fmoles/mg of protein. *Kappa* sites have been determined in two tumors: they represented 25% and 80% of the total number of high-affinity sites. Because of the relative abundance of *kappa* sites on pheochromocytoma membranes, a preliminary pharmacological characterization was undertaken in Case 1, which was the one completely analyzed (Fig. 4). The *kappa* nature of these sites has been confirmed by the fact that EKC and cyclazocine were the most active substances (K_I 0.71 and 0.92 nM, respectively). They were followed by naloxone (2.8 nM), ketocyclazocine (4.9 nM), and morphine (39 nM); fentanyl, an almost pure *mu* agonist, showed a $K_I > 310$ nM. Similar K_I values for EKC, ketocyclazocine, cyclazocine, and fentanyl were found by Audigier *et al.* (7) for guinea pig spinal cord *kappa* sites, whereas naloxone and morphine were much more active on the human pheochromocytoma studied.

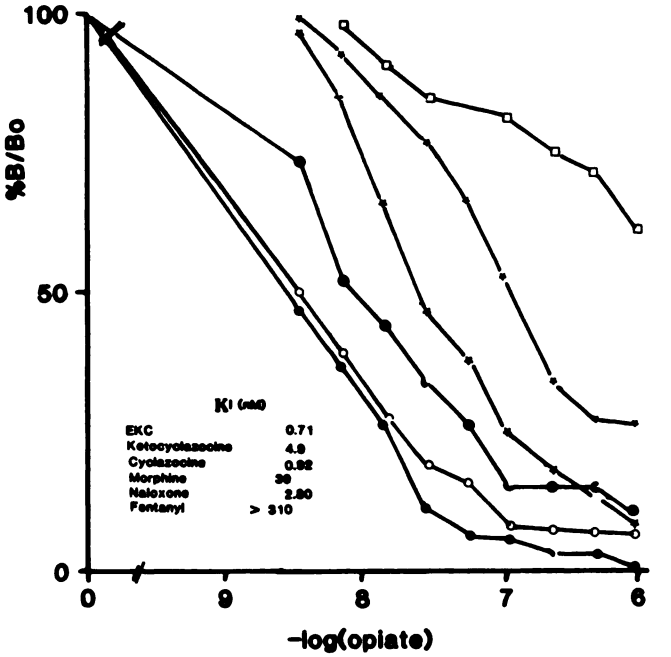

FIG. 4. *Displacement of [^{3}H]EKC bound on a human pheochromocytoma membrane preparation by different opiates in the presence of 5 μM DADLE*

Displacement of tritiated EKC by (●), ketocyclazocine (★), cyclazocine (○), morphine (☆), naloxone (⊕) and Fentanyl (□). Binding experiments were performed as described in the text. K_I values for each ligand were calculated according to the formula of Cheng and Prusoff (12) $K_I = IC_{50}/(1 + S/K_D)$, in which K_D = the dissociation constant of [^{3}H]EKC (0.980 nM) and S = the concentration of [^{3}H]EKC in the assay (2.3 nM).

DISCUSSION

Chromaffin cells represent a simple model for studying biosynthetic and release mechanisms of the nervous system. Although much work has recently been done in the field of biosynthetic and exocytotic mechanisms (see ref. 1 for a review), little is known about the action of opioids on these cells. These studies indicate that opioids could be modulators of basal and/or evoked cell secretion. This action is stereoselective and reversed by opioid antagonists, indicating that opioid receptor(s) must be involved. Furthermore, pheochromocytomas and bovine adrenal medullas contain and secrete considerable amounts of endogenous opioid peptides (2, 3). With the exception of the original paper by Chavkin *et al.* (4) indicating stereoselective, saturable binding on bovine chromaffin cell membranes, very few other papers have dealt with the characterization of opiate binding sites in this tissue (5, 13). It was therefore of interest to characterize opioid receptors on adrenal medullary membranes of human and animal origin.

Our data show stereoselective and saturable binding of opiate ligands to bovine membranes, confirming previous work (4, 5), and reveal opiate binding sites on membranes from human pheochromocytomas. Association and dissociation kinetics show that steady state is achieved in both tissues after 25 min of incubation.

In a recent study, Saiani and Guidotti (5) examined the binding of different opioids on bovine adrenomedullary membranes. Comparison with the results presented in this study reveals great discrepancies: in our hands etorphine showed a 9-fold increase in affinity while identifying only half as many sites. EKC, sharing the same affinity in both studies, identified, in the present study, 6 times more sites. DADLE binding presented a 3 times lower affinity in our hands, but identified three times more sites than in the study by Saiani and Guidotti.

TABLE 5

[^{3}H]EKC binding parameters on membranes of human pheochromocytomas: effect of DADLE

Membrane preparation and binding conditions are described under Materials and Methods and in the legend to Fig. 2. DADLE concentration was 5 μM. Binding characteristics were determined from Scatchard plots. Assays were run in triplicate with intra-assay coefficient of variation <10% (± 2 SD).

Case	Tritiated ligand + effector	High-affinity sites		Low-affinity sites	
		B_{max}	K_D	B_{max}	K_D
		fmoles/mg protein	*nM*	*fmoles/mg protein*	*nM*
1	EKC + 0	163	1.12	275	4.15
	EKC + DADLE	41	0.98	114	6.72
2	EKC + 0	70	0.37	206	5.60
3	EKC + 0	30	0.78	333	20.40
4	EKC + 0	81	1.13	304	10.04
	EKC + DADLE	65	0.72	291	30.84

Methodological differences during membrane preparation could be responsible for the observed discrepancies. (a) In our study a hypoosmotic medium was used throughout the whole preparation, whereas Saiani and Guidotti (5) used isoosmotic conditions followed by hypoosmotic lysis in distilled water. (b) The attempt to remove endogenous opioid peptides in our study by preincubation in assay buffer for 30 min at 37° could account for the observed discrepancies. Chromaffin cells contain large amounts of opioid peptides which, liberated during cell lysis, could mask a number of opioid receptors. (c) Assuming that adrenal opioid receptors possess the same characteristics as those found in the central nervous system, preincubation in Tris-HCl buffer without the addition of divalent ions could diminish the number of binding sites, as reported by Sadee *et al.* (14), and thus account for the decreased number of etorphine sites reported in the present study.

The number and affinity of *mu* sites reported in the present study on bovine membranes (21–26 fmoles/mg of protein, or 2.1–2.6 pmoles/g of tissue, taking into consideration a yield of 1% in crude membrane preparations) are comparable with the number of binding sites reported by Chavkin *et al.* (4). Furthermore, the number of DADLE sites found in the present study on bovine membranes is comparable to that reported by Kumakura *et al.* (13) on isolated chromaffin cell membrane preparations.

Saiani and Guidotti (5) consider DADLE and EKC to be pure markers of one class of opiate sites (*delta* and *kappa*, respectively). Recent work indicates that neither ligand is a pure marker of one class of receptors (see ref. 8 for a discussion). Hence, EKC can identify, in addition to *kappa* sites, *delta*, *mu*, and benzomorphan sites. DADLE identifies *delta* and *mu* sites with a great affinity, provoking the inflections observed in the competition curves by [^{3}H]etorphine and [^{3}H]EKC binding; when used at high concentrations, it can also mask the benzomorphan receptor (Fig. 3) (see also ref. 6). Therefore, receptor type characterization necessitates a specific masking of some receptor subtypes during saturation or competition experiments. Two methods of receptor masking exist: (a) providing saturation concentrations of an effector which masks one or more receptor subtypes; (b) introducing in the assay a constant ratio of effector to radioligand [this method has been used by Gillian and Kosterlitz (8) for the determination of opioid receptor subtypes in rat brain]. The first method was used in the present study. Morphiceptin, a pure *mu* agonist; DSLET, a pure *delta* agonist; and saturating concentrations of DADLE were used as effectors. The spectrum of opioid receptor types found showed a preponderance of benzomorphan sites representing about 70% of total high-affinity sites.

To our knowledge, the present study is the first attempt to characterize opioid receptors on pheochromocytoma membranes. In these tumors a great heterogeneity was found concerning the number and affinity of opioid receptors, as well as their relative distributions [at least for the *kappa* subtype (Table 5)]. It was of interest to compare the opioid receptor spectrum between a human tumor, for which sufficient material was obtained at surgery, and normal tissue from another species (bovine adrenal medulla). Several differences have been observed:

1. An absence of *delta* sites on human membranes. Different hypotheses could explain this result: for example, the huge amounts of opioid peptides contained and secreted by this pheochromocytoma (5 μg/g of tissue M-Enk-IR) could provoke a down-regulation of the *delta* receptor, as reported by Chang *et al.* (15) in the case of cultured neuroblastoma cells. Another possibility could be the extreme sensitivity of *delta* receptors to freezing, since an almost complete abolition of *delta* sites after freezing has been observed on bovine membranes (data not shown).
2. A decrease in the affinity of *mu* sites on human membranes. *Mu* sites represent ≃20% of total high-affinity receptors on bovine membranes, but they represent only 9% of sites on membranes of human origin.
3. An equal distribution of benzomorphan sites in both tissues (64% on bovine and 68% on human membranes).
4. A greater density of *kappa* sites on human membranes. They represent 25% of the high-affinity sites in one pheochromocytoma, and 80% of the high-affinity sites on a second tumor (Table 5); bovine membranes possess 10% *kappa* sites.

The observed differences could be attributed to either species (human versus bovine tissues) or tumor differences.

In the present study only the high-affinity component of [^{3}H]EKC has been examined. This radioligand presents, in addition to the high-affinity, a lower-affinity component in both types of membranes which is partially displaceable by DADLE (see Tables 2 and 3). Although no attempt was made to characterize this low-affinity interaction of [^{3}H]EKC with membranes, evidence exists that this site must not represent an "opioid" receptor as benzodiazepines and ethyl-β-carboline compete for this [^{3}H]EKC binding with high affinity (16).

The major finding reported in the present study is the preponderance on adrenal medullary membranes of bovine and humans of a large number of *kappa* and benzomorphan sites which represent more than 80% of the total high-affinity sites detected. No endogenous opioid peptide was tested in the present study. Others (6, 7, 17) have found that dynorphin-related peptides and M-Enk-Arg^6-Phe^7 are the preferential, although not specific, ligands for the *kappa* and benzomorphan receptors, respectively. Both peptides were found in the adrenal medulla. They could therefore be putative candidates for the regulation of chromaffin cell secretion.

REFERENCES

1. Livett, B. G., P. Boksa, D. M. Dean, F. Mizobe, and M. H. Lindenbaum. Use of isolated chromaffin cells to study basic release mechanisms. *J. Auton. Nerv. Syst.* **7**:59–86 (1983).
2. Wilson, S. P., J. W. Lewis, and O. H. Viveros. Interplay between opioid peptides and catecholamines in adrenal medulla and human pheochromocytoma, in *Molecular Pharmacology of Peripheral Catecholaminergic System.* Ibiza, Spain, Abstr. 37 (1982).
3. Castanas, E., P. Giraud, C. Oliver, B. Conte-Devolx, F. Boudouresque, M. Orlando, P. E. Chabrier, L. Eiden, F. Cesselin, and G. Gunz. Opiate peptides: Biosynthetic and pharmacological studies in primary cell cultures, in *Molecular Pharmacology of the Peripheral Catecholaminergic System.* Ibiza, Spain, Abstr. 45 (1982).
4. Chavkin, C., B. M. Cox, and A. Goldstein. Stereospecific opiate binding in bovine adrenal medulla. *Mol. Pharmacol.* **15**:751–753 (1980).

5. Saiani, L., and A. Guidotti. Opiate receptor-mediated inhibition of catecholamine release in primary cultures of bovine adrenal chromaffin cells. *J. Neurochem.* **39**:1669–1676 (1982).
6. Attali, B., C. Guarderes, H. Mazarguil, Y. Audigier, and J. Cros. Evidence for multiple "kappa" binding sites by use of opioid peptides in the guinea pig lumbosacral spinal cord. *Neuropeptides* **3**:53–64 (1982).
7. Audigier, Y., B. Attali, C. Guarderes, H. Mazarguil, and J. Cros. Multiplicity of kappa opiate sites in the central nervous system, in *Veterinary Pharmacology and Toxicology* (Y. Ruckebusch, J. Toutain, and H. Koritz, eds.). MTP Press, 451–463 (1983).
8. Gillian, M. G. C., and H. W. Kosterlitz. Spectrum of the μ, δ and κ binding sites in homogenates of rat brain. *Br. J. Pharmacol.* **77**:461–469 (1982).
9. Chang, K. J., E. Hazoum, and P. Cuatrecasas. Novel opiate binding sites selective for benzomorphan drugs. *Proc. Natl. Acad. Sci. U. S. A.* **78**:4141–4145 (1981).
10. Chang, K. J., A. Killian, E. Hazoum, P. Cuatrecasas, and J. K. Chang. Morphiceptin (NH_4-Tyr-Pro-Phe-Pro-$CONH_2$): a potent and specific agonist for morphine (μ) receptors. *Science* (*Wash. D. C.*) **212**:75–77 (1981).
11. Pasten, I. H., M. C. Fournié-Zalouski, and B. P. Roques. Tyr-D-Ser-Gly-Phe-Leu-Thr, a highly preferential ligand for δ opiate receptors. *F. E. B. S. Lett.* **118**:245–247 (1980).
12. Cheng, Y. C., and W. H. Prusoff. Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 percent inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
13. Kumakura, K., A. Guidotti, H. Y. Yang, L. Saiani, and E. Costa. A role for the opiate peptides that presumably coexist with acetylcholine in splachnic nerves. *Adv. Biochem. Pharmacol.* **22**:571–580 (1980).
14. Sadee, W., A. Pfeiffer, and A. Herz. Opiate receptor: multiple effects of metal ions. *J. Neurochem.* **39**:659–667 (1982).
15. Chang, K. J., R. W. Eckel, and S. G. Blanchard. Opioid peptides induce reduction of enkephalin receptors in cultured neuroblastoma cells. *Nature (Lond.)* **296**:446–448 (1982).
16. Attali, B., C. Guarderes, Y. Audigier, and J. Cros. Unusual binding properties of [^{3}H]ethylketocyclazocine low affinity sites in guinea-pig CNS. *International Narcotic Research Conference*. Garmisch-Partenkirchen FRG, P-153 (1983).
17. Corbett, A. D., S. J. Paterson, A. I. McKnight, J. Magnan, and H. W. Kosterlitz. Dynorphin 1-8 and dynorphin 1-9 are ligands for the K-subtype of opiate receptor. *Nature* **299**:79–81 (1982).

Send reprint requests to: Dr. E. Castanas, Laboratoire de Médecine Expérimentale, ERA 982 CNRS, UER Nord, Bd P. Dramard F 13326 Marseille Cedex 15, France.