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OPIATE RECEPTOR BINDING PROFILE IN THE RABBIT CEREBELLUM AND BRAIN MEMBRANES

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Abstract—The equilibrium dissociation constants (K_d) and maximal binding capacities of tritiated dihydromorphine (DHM), [D-Ala^2,D-Leu^5]enkephalin (DADLE), ethylketocyclazocine (EKC) and human β-endorphin (β-EP) in rabbit cerebellum and brain membranes have been investigated. Binding of tritiated DHM and DADLE was adequately described by a single affinity class of binding sites, while that of EKC required two affinity sites. Binding of tritiated β-EP was also consistent with a single affinity class of binding sites by Scatchard analysis, but inhibition of the binding with type selective opiate receptor ligands revealed multiple sites. Sequential displacement of a broad spectrum opiate ligand, diprenorphine (DIP), by type selective ligands showed that cerebellum membranes are relatively rich in μ (40%) and deficient in κ (12%) binding sites, while brain membranes are relatively rich in κ (32%) and deficient in μ (12%) binding sites. β-EP displaces 88 and 73% of tritiated DIP from cerebellum and brain membranes, respectively, suggesting multiple sites of β-EP binding.

The existence of multiple opiate receptors in the mammalian brain has been well documented [1–5]. However, there are few studies on the opiate binding sites in cerebellum of mammals except lagomorphs [6, 7]. We recently reported that rabbit cerebellum possesses abundant high affinity binding sites for β-EP† [8]. In view of the unique function and neuronal architecture of cerebellum, it is of interest to determine the binding characteristics of various opiate receptors in this tissue. The present study was conducted to disclose the spectrum of μ, δ, κ and β-EP sites and their binding profiles in rabbit cerebellum and brain.

MATERIALS AND METHODS

Labeled compounds

[^{3}H]-β-EP (50 Ci/mmole) was prepared by catalytic reduction of the appropriate iodinated synthetic analog as described previously [9]. [^{3}H]DHM (72 Ci/mmole), [^{3}H]DADLE (44 Ci/mmole), and [^{3}H]EKC (16 Ci/mmole) were from New England Nuclear. [^{3}H]DIP (7.5 Ci/mmole) was from Amersham/Searle. Purity of these ^{3}H-ligands was checked using reverse phase HPLC.

Unlabeled compounds and reagents

β-EP was a synthetic product [10]. Morphine sulfate was from Mallinckrodt and DADLE from Sigma; EKC and U-50488 were gifts from Sterling-Winthrop and the Upjohn Co. respectively. BSA was from Schwarz/Mann and bacitracin from Sigma. Myelin basic protein was isolated from bovine brain as described [11].

Membranes

Cerebellum and brain were dissected from 5 to 7 lb male New Zealand rabbits, homogenized with a polytron (Brinkmann PT-20) (setting 6.5, 20 sec) in 10 vol. of Tris–HCl buffer (50 mM, pH 7.4), centrifuged at 15,000 *g* for 30 min, and washed as described previously [8]. Washed membranes were stored at −70° at a concentration of 5 mg protein/ml in 20% glycerol, 50 mM Tris–HCl, pH 7.4. Membrane protein was estimated by the method of Lowry *et al.* [12].

Binding experiments

All binding experiments were carried out in plastic tubes with an assay buffer consisting of 0.1% BSA, 0.01% bacitracin, and 50 mM Tris–HCl at pH 7.4. The order of addition of reagents to tubes was assay buffer, competing ligands (if any), the labeled ligand and membranes. In most experiments, 0.5 mg of the membrane protein was incubated in a final volume of 2.0 ml for 60 min at 23°. Binding was terminated by rapid vacuum filtration through Whatman GF/B glass fiber filters. The filters were then washed twice with 5 ml of ice-cold washing buffer (assay buffer without bacitracin), transferred to vials containing 3 ml of scintillation fluid, and allowed to stand overnight at 23°. When using [^{3}H]-β-EP as primary ligand, the GF/B filters were pretreated with myelin basic protein as described previously [13, 14]. Radioactivity was measured by liquid scintillation counting. Specific binding was determined by the difference in radioactivity trapped on filters in the presence and absence of a 1.0 μM concentration of the corresponding unlabeled ligand.

Sequential displacement was performed by meas-

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† Abbreviations: β-EP, β-endorphin; DHM, dihydromorphine; DADLE, [D-Ala^2, D-Leu^5]enkephalin; EKC, ethylketocyclazocine; β_h-EP, human β-endorphin; DIP, diprenorphine; [^{3}H]-β-EP, [$^3H_2$-Tys^{27}]-β_h-EP; HPLC, high performance liquid chromatography; and BSA, bovine serum albumin.

Table 1. Affinity and capacity of binding for opioid peptides and opiates in rabbit cerebellar and brain membrains

Ligand	K_d (nM)		Binding capacity (fmoles/mg protein)	
	Cerebellum	Brain	Cerebellum	Brain
DHM	0.52 ± 0.07*	0.85 ± 0.06	230 ± 16.4	66.9 ± 5.2
DADLE	2.4 ± 0.17	1.7 ± 0.31	179.5 ± 38.4	139 ± 5.3
EKC†	0.24 ± 0.1	1.2 ± 0.1	223.6 ± 7.2	206 ± 10.2
β-EP	0.28 ± 0.03	0.45 ± 0.06	350 ± 7.8	271.7 ± 9.3

* Values are means ± S.D.
† Only high affinity site is shown.

uring the binding of [^{3}H]DIP in the absence and in the presence of 100 nM morphine, 100 nM morphine plus 1 μM DADLE, 100 nM morphine plus 1 μM DADLE plus 100 nM EKC, and 100 nM β-EP. The fraction which was displaced by morphine was taken as the μ site. The further displacement achieved by the addition of DADLE was assumed to represent the δ site. The difference between displacement with morphine + DADLE and with morphine + DADLE + EKC was assumed to represent the κ site. Nonspecific binding was defined as binding remaining in the presence of morphine, DADLE, EKC, and β_h-EP together at the concentration given above. This method was suggested by Pfeiffer *et al.* [15] to estimate receptor type densities in human brain. Since DADLE is not very specific for δ sites, the estimate for δ site density is obtained from differences in binding in the presence of morphine, with or without DADLE, rather than from DADLE alone. The estimate for κ site density is also obtained by differences in binding. This approach avoids the problem of lack of ligand selectivity by first using the most selective ligand alone, then in combination with a less selective ligand.

Data analysis

Saturation experiments were analyzed by unweighted linear least squares regression of the ratio of specific binding to free labeled ligand against specific binding of the ligand (Scatchard analysis). Initial estimates for nonlinear Scatchard plots were obtained as described [16]. Competition experiments were analyzed by weighted nonlinear least squares analysis of a four parameter (IC_{50}, slope, minimal and maximal bound) logistic model. Some of the ligands showed heterogeneous curves in competition experiments, as indicated by shallow slopes and poor fit to a four parameter logistic model. In particular, the displacement curve of [^{3}H]DHM and [^{3}H]EKC by β-EP in cerebellum was characterized by two distinct components. For these two curves,* the apparent K_i was calculated from the IC_{50} of the lower portion of the curve. This was computed by the least squares method as described above, taking the plateau region to be maximal binding. The apparent affinity obtained is a function of receptor density as well as receptor affinity.

RESULTS

Binding characteristics obtained by saturation analysis

Table 1 presents the K_d values and binding capacity of various tritiated ligands in rabbit cerebellum and brain. Binding of [^{3}H]DHM and [^{3}H]DADLE was adequately described by a single affinity class of sites by Scatchard analysis. The binding of [^{3}H]EKC was found to be multiphasic (Fig. 1); only the high affinity site is shown in Table 1. Binding of [^{3}H]-β-EP was monophasic; however, binding site heterogeneity was revealed by kinetics of dissociation [8] and by inhibition of binding by prototype opiate ligands (see Figs. 2 and 5). The apparent affinity of DHM, EKC, and β-EP was higher in cerebellum than in brain. Cerebellum also had a larger binding capacity than brain, most prominently for DHM (μ opiate receptor).

Characterization of binding sites by competition experiments

[^{3}H]DHM. In cerebellum, [^{3}H]DHM labeled a population of binding sites which could be displaced completely by EKC and β-EP, and nearly completely displaced (98% inhibition) by DADLE at a concentration of 1 μM, as shown in Fig. 2A. U-50488, a compound reported to be a specific κ ligand [18], showed weak but complete inhibition of [^{3}H]DHM binding at a concentration of 40 μM- β-EP inhibited [^{3}H]DHM binding with the highest potency. Its completion curve was biphasic, with a plateau at 65% inhibition. The apparent inhibition constant (K_i) of the higher affinity phase was estimated to be lower than 0.1 pM. This apparent high affinity site for β-EP had not been detected by saturation analysis and could be due to the slow dissociation of β-EP from the binding site as has been reported previously [8]. The apparent K_i values of all ligands tested are given in Table 2. The value for morphine agrees well with its apparent K_d obtained from saturation analysis. In brain all ligands, including β-EP, showed a simple, monophasic competition curve (Fig. 2B). The order of potency is similar to that in cerebellar membranes (i.e. β-EP > EKC > morphine > DADLE > U-

* Analysis of these curves and others in this data set by the model of mass action as implemented by Munson and Rodbard [17] has not, to date, proven possible (the data appear to be inadequately fit by this model). For this reason, analysis of relative affinities by IC_{50} is retained.

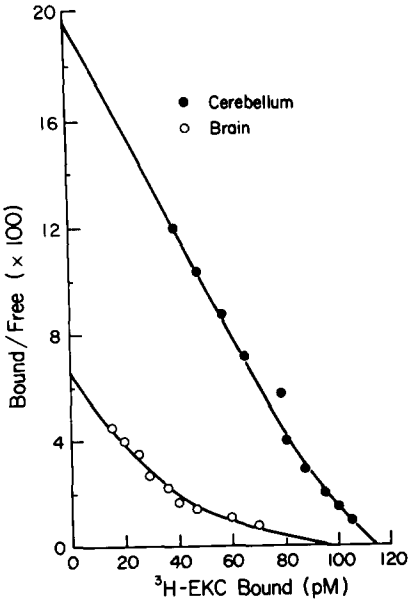

Fig. 1. Saturation analysis of binding of [^{3}H]EKC to cerebellar (●) and brain (○) membranes of the rabbit. Data shown are from one of three replicate experiments. In cerebellar membranes, the K_d and binding capacity are 0.24 nM and 208 fmoles/mg for the high affinity site and 1.27 nM and 235 fmoles/mg for the low affinity site. In brain membrane, the K_d and binding capacity are 0.95 nM and 229 fmoles/mg for the high affinity site, and 3.1 nM and 381 fmoles/mg for the low affinity site respectively.

50488). However, all ligands had lower affinity (higher K_i value) in brain (Table 2), and DADLE and U-50488 did not completely displace [^{3}H]DHM binding at concentrations of 1.0 and 40 μM respectively. It is interesting to find that EKC, a compound claimed to be a κ specific ligand, showed a three to four times higher potency than morphine in displacing [^{3}H]DHM.

[^{3}H]DADLE. As summarized in Table 3, the apparent K_i for DADLE calculated from competition experiments was 2.0 ± 0.2 in cerebellum and 1.7 ± 0.3 in brain membranes. These values agree well with those obtained from saturation experiments. Opioid peptides and opiates displaced [^{3}H]-DADLE binding in cerebellar membranes (Fig. 3) with a relative potency similar to that found in displacement of [^{3}H]DHM (Table 3). In brain membrane, however, EKC and morphine showed a marked increased in apparent K_i values so that the order of potency changed to β-EP > DADLE > EKC > morphine > U-50488. Morphine and U-50488 were unable to completely displace [^{3}H]-DADLE binding in both cerebellum and brain membranes (Fig. 3). The lower apparent affinity of DHM and EKC in brain may be attributed in part to the relatively low content of the μ binding site in brain (see below) to which DHM and EKC bind with high affinity.

[^{3}H]EKC. The opiate binding sites labeled by [^{3}H]-EKC could not be displaced completely by all other ligands tested. Even β-EP showed a flat, incomplete

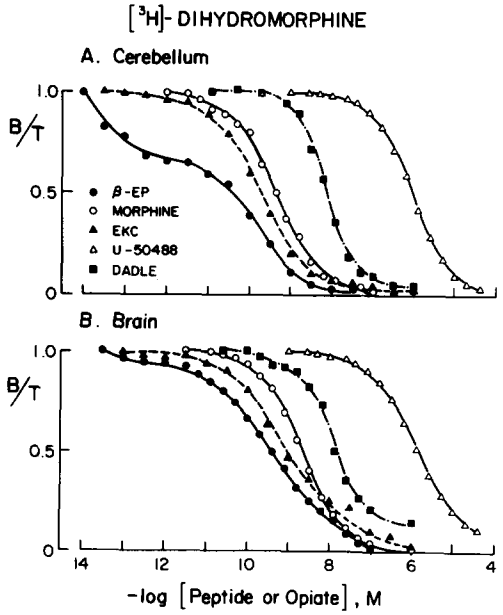

Fig. 2. Inhibition of [^{3}H]dihydromorphine binding to rabbit cerebellar (A) or brain (B) membranes. The fractopin of ^{3}H-label retained on filters after subtraction of nonspecific binding is plotted on the ordinate. Data shown are from one of three replicate experiments. Each dose of each ligand was tested in duplicate, and the responses were averaged.

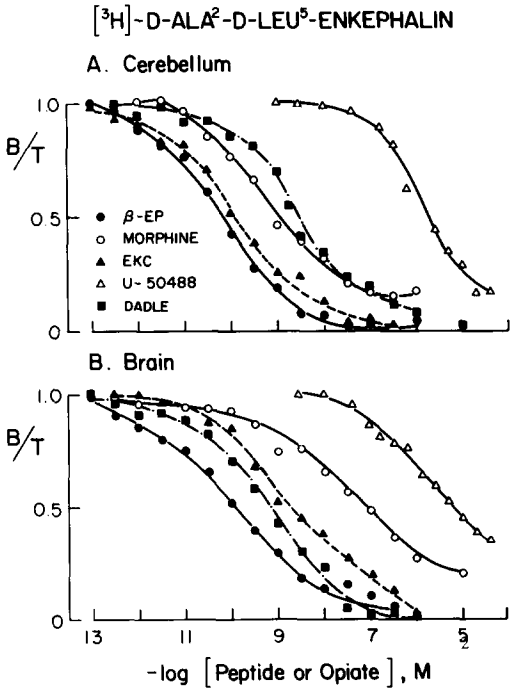

Fig. 3. Inhibition of tritested [D-Ala^2,D-Leu^5]enkephalin binding to rabbit cerebellar (A) or brain (B) membranes. The fraction of ^{3}H-label retained on filters after subtraction of nonspecific binding is plotted on the ordinate. Data shown are from one of three replicate experiments. Each dose of each ligand was tested in duplicate, and the responses were averaged.

Table 2. Apparent binding affinity of opioid peptides and opiates in rabbit cerebellar and brain membranes against [^{3}H]dihydromorphine

Unlabeled compound	Cerebellum		Brain		K_i ratio (brain/cerebellum)
	K_i* (nM)	Relative potency	K_i (nM)	Relative potency	
β-Endorphin	0.03 ± 0.01†	100	0.18 ± 0.02	100 (16.7)‡	6.0
Morphine	0.46 ± 0.06	6.5	0.96 ± 0.07	18.8 (3.1)	2.1
DADLE	1.29 ± 0.21	2.3	3.78 ± 0.59	4.8 (0.8)	2.9
EKC	0.11 ± 0.02	27.3	0.33 ± 0.09	54.5 (9.1)	3.0
U-50488	496 ± 7	0.006	588 ± 32	0.03 (0.005)	1.2

* K_i was calculated according to the equation $K_i = \frac{IC_{50}}{1 + F/K_d}$, where F is the concentration of free labeled ligand and K_d is the apparent dissociation constant of labeled ligand.
† Values are means ± S.D.
‡ Potency relative to β-endorphin in cerebellum.

Table 3. Apparent binding affinity of opioid peptides and opiates in rabbit cerebellar and brain membranes against treated [D-Ala^2,D-Leu^5]enkephalin

Unlabeled compound	Cerebellum		Brain		K_i ratio (brain/cerebellum)
	K_i* (nM)	Relative potency	K_i (nM)	Relative potency	
β-Endorphin	0.07 ± 0.02†	100	0.02 ± 0.01	100 (350)‡	0.3
Morphine	0.40 ± 0.02	17.5	3.33 ± 1.40	0.6 (2.1)	8.3
DADLE	2.01 ± 0.18	3.5	1.67 ± 0.28	1.2 (4.2)	0.8
EKC	0.26 ± 0.08	26.9	2.50 ± 0.16	0.8 (2.8)	9.6
U-50488	374 ± 33	0.02	627 ± 50	0.003 (0.01)	1.7

* K_i was calculated according to the equation $K_i = \frac{IC_{50}}{1 + F/K_d}$, where F is the concentration of free labeled ligand and K_d is the apparent dissociation constant of labeled ligand.
† Values are means ± S.D.
‡ Potency relative to β-endorphin in cerebellum.

Table 4. Apparent binding affinity of opioid peptides and opiates in rabbit cerebellar and brain membranes against [^{3}H]ethylketocyclazocine

Unlabeled compound	Cerebellum		Brain		K_i ratio (brain/cerebellum)
	K_i* (nM)	Relative potency	K_i (nM)	Relative potency	
β-Endorphin	0.05 ± 0.01†	100	0.36 ± 0.04	100 (14)‡	7.2
Morphine	1.25 ± 0.10	4	22.6 ± 4.1	1.6 (0.2)	18.1
DADLE	3.01 ± 0.38	1.7	19.2 ± 2.8	1.9 (0.3)	6.4
EKC	0.22 ± 0.04	22.7	0.96 ± 0.25	37.5 (5.2)	4.4
U-50488	475 ± 89	0.01	206 ± 10	0.2 (0.02)	0.4

* K_i was calculated according to the equation $K_i = \frac{IC_{50}}{1 + F/K_d}$, where F is the concentration of free labeled ligand and K_d is the apparent dissociation constant of labeled ligand.
† Values are means ± S.D.
‡ Potency relative to β-endorphin in cerebellum.

displacement curve (Fig. 4), although it could inhibit [^{3}H]EKC binding at very low concentrations in cerebellum membrane. The order of potency to inhibit [^{3}H]EKC binding was similar to that against [^{3}H]-DHM and [^{3}H]DADLE in cerebellum, while in brain morphine was about the same or even less potent than DADLE (Table 4). Comparing the K_i ratios in Table 4, U-50488 was found to be the only ligand that showed higher potency to displace [^{3}H]EKC in brain than in cerebellum. Considering the relatively higher content of κ sites in brain, this result seems to support the finding of Vonvoigtlander *et al.* [18] that U-50488 is a more selective κ agonist.

[^{3}H]-β-EP. We have shown previously that %[^{3}H]-

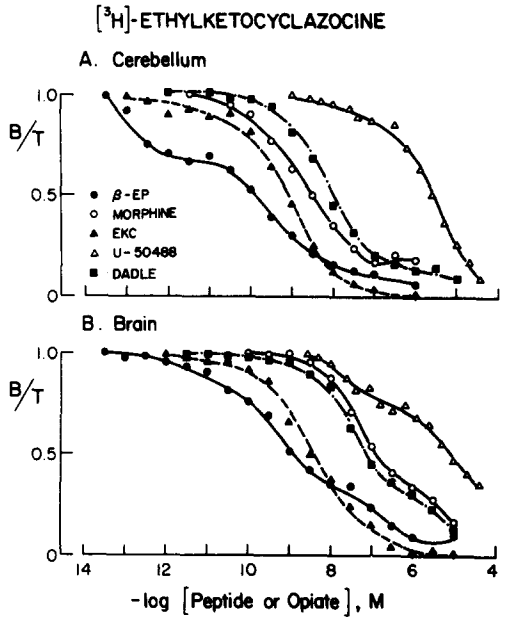

Fig. 4. Inhibition of [^{3}H]ethylketocyclazocine binding to rabbit cerebellar (A) or brain (B) membranes. The fraction of ^{3}H-label retained on filters after subtraction of nonspecific binding is plotted on the ordinate. Data shown are from one of three replicate experiments. Each dose of each ligand was tested in duplicate, and the responses were averaged.

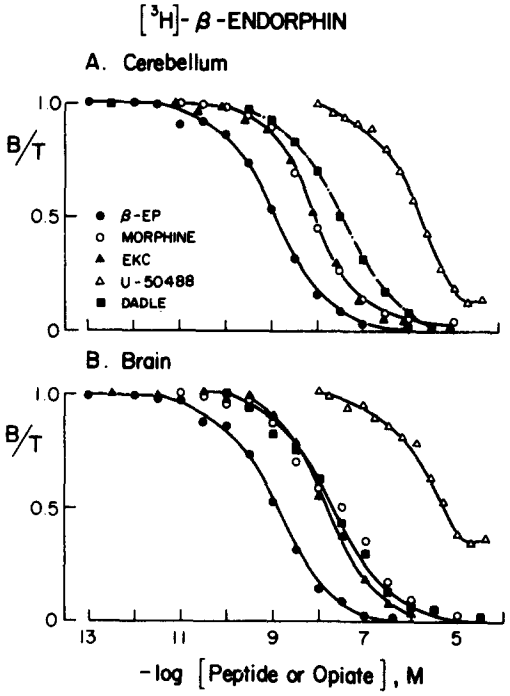

Fig. 5. Inhibition of [^{3}H]-β-endorphin binding to rabbit cerebellar (A) or brain (B) membranes. The fraction of ^{3}H-label retained on filters after subtraction of nonspecific binding is plotted on the ordinate. Data shown are from one of three replicate experiments. Each dose of each ligand was tested in duplicate, and the responses were averaged.

β-EP can bind a large number of binding sites, amounting to 350 ± 8 fmoles/mg protein in cerebellum and 272 ± 9 fmoles/mg protein in brain [8]. The apparent K_d values determined by saturation experiments in cerebellum and brain were 0.28 and 0.45 nM respectively. Competition studies in cerebellum revealed that morphine and EKC had similar potencies in displacing [^{3}H]-β-EP binding (K_i = 1.71 and 1.96 respectively), while DADLE was about two times less potent than morphine (Fig. 5B and Table 5). In brain membranes, morphine, DADLE and EKC showed similar apparent K_i values against [^{3}H]-β-EP (Fig. 5B and Table 5), while U-50488 displaced with a K_i value as high as 959 nM. It is surprising that morphine could displace more than 90% and DADLE completely displace β-EP binding in both brain and cerebellum, although the number of binding sites for DHM or DADLE determined by Scatchard analysis was always less than for β-EP.

Relative abundance of opiate receptors estimated by sequential displacement

It is more informative to know the relative abundance of different opiate receptors than just to know

Table 5. Apparent binding affinity of opioid peptides and opiates in rabbit cerebellar and brain membranes against [^{3}H]-β-endorphin

Unlabeled compound	Cerebellum		Brain		K_i ratio (brain/cerebellum)
	K_i* (nM)	Relative potency	K_i (nM)	Relative potency	
β-Endorphin	0.24 ± 0.05†	100	0.48 ± 0.05	100 (50)‡	2.0
Morphine	1.71 ± 0.05	14	4.77 ± 0.24	10 (5)	2.8
DADLE	3.57 ± 0.09	6.7	5.57 ± 0.3	8.6 (4.3)	4.4 2
EKC	1.96 ± 0.18	12.2	4.0 ± 0.48	12 (6.0)	2.0
U-50488	359 ± 49	0.07	959 ± 154	0.05 (0.03)	2.7

* K_i was calculated according to the equation $K_i = \frac{IC_{50}}{1 + F/K_d}$, where F is the concentration of free labeled ligand and K_d is the apparent dissociation constant of labeled ligand.

† Values are means ± S.D.

‡ Potency relative to β-endorphin in cerebellum.

Table 6. Relative abundance of opiate receptors in rabbit cerebellum and brain estimated by sequential displacement of [^{3}H]diprenorphine

	Percentage of total [^{3}H]diprenorphine bound					Total bound¶ (fmoles/mg protein)
	μ*	†	‡	Others§	β-EP‖	
Cerebellum	40 ± 5**	37 ± 1	12 ± 1	11	88 ± 3	534 ± 14
Brain	12 ± 1	37 ± 3	32 ± 3	18	73 ± 9	310 ± 11

* Measured by displacement with 100 nM morphine.
† Measured by the difference between displacement with 100 nM morphine and with 100 nM morphine + 1000 nM DADLE.
‡ Measured by the difference between displacement with 100 nM morphine + 100 nM DADLE and with 100 nM morphine + 1000 nM DADLE + 100 nM EKC.
§ Measured by remainder from the displacement with morphine + DADLE + EKC at the concentrations given above.
‖ Measured by displacement with 100 nM β_h-EP.
¶ Nonspecific binding is defined as binding remaining in the presence of morphine, DADLE, EKC and β_h-EP together at the concentrations given above, and has been subtracted.
** Values are means ± S.D.

the capacity of binding. Using sequential displacement of [^{3}H]DIP (an opiate ligand reported to bind all types of opiate receptors in brain [19], the relative content of μ, δ and κ receptors was estimated to be 40, 37, and 12% respectively, in cerebellum and 12, 37 and 32, respectively, in brain (Table 6). β-EP displaced 88% of [^{3}H]DIP binding in cerebellum and 73% in brain; these values appear to equal the sum of displacement of all three types of receptors (μ, δ and κ). The relative deficiency in μ receptors in brain is consistent with the data obtained from saturation analysis (see Table 1). The relative content (%) of μ and δ receptors in cerebellum and brain obtained by either saturation analysis or sequential displacement is fairly close. Values of 42% for μ, 33% for δ sites in cerebellum and 19% for μ, 39% for δ sites in brain were obtained by dividing the maximal binding capacity (Table 1) by the total [^{3}H]DIP bound. Due to the rather broad spectrum of EKC binding, the capacity of its high affinity site shown in Table 1 probably represents the sum of its binding to κ as well as μ sites. High affinity binding of EKC to μ and κ sites has been shown in this (see Tables 3 and 4) and other reports [20, 21].

Effect of $Na^{\pm}$ *and low temperature on* [^{3}*H*]*EKC binding*

It has been reported that low temperature and Na^+ inhibit binding of [^{3}H]EKC to μ and δ receptors but enhance binding to κ receptor [22]. Therefore, one would expect binding to decrease in brain areas rich in μ and δ, and to increase in areas rich in κ and deficient in μ on adding Na^+. At 23°, 100 mM Na^+ reduced [^{3}H]EKC binding to 48% of control in cerebellum but increased binding to 110% of the control in brain. At 5°, 100 mM Na^+ reduced [^{3}H]EKC binding to 17.5% in cerebellum and 32.2% in brain, relative to control (23°, no Na^+). These values are similar to those determined by sequential displacement (Table 6).

DISCUSSION

The maximal binding capacity and relative content of various types of opiate binding sites differ between rabbit brain and cerebellum as shown by both saturation and sequential displacement methods. Cerebellum is rich in μ and δ and deficient in κ while brain is rich in κ and δ but deficient in μ. These

Table 7. Apparent binding affinity of opioid peptides and opiates in rabbit cerebellar and brain membranes from competition experiments

	K_i in cerebellum (nM)				K_i in brain (nM)			
	Labeled				Labeled			
Unlabeled	β_h-EP	DHM	DADLE	EKC	β_h-EP	DHM	DADLE	EKC
β-EP	0.24 (±0.05)	0.03 (±0.01)	0.07 (±0.02)	0.05 (±0.01)	0.48 (±0.04)	0.18 (±0.02)	0.02 (±0.01)	0.36 (±0.04)
Morphine	1.71 (±0.05)	0.46 (±0.06)	0.4 (±0.2)	1.25 (±0.1)	4.77 (±0.24)	0.96 (±0.07	3.33 (±1.4)	22.6 (±4.10)
DADLE	3.57 (±0.09)	1.29 (±0.21)	2.01 (±0.18)	3.01 (±0.38)	5.57 (±0.3)	3.79 (±0.59)	1.67 (±0.28)	19.2 (±2.8)
EKC	1.96 (±0.18)	0.11 (±0.02)	0.26 (±0.08)	0.22 (±0.04)	4.00 (±0.48)	0.33 (±0.09)	2.50 (±0.16)	0.96 (±0.25)
U-50488	395 (±49	374 (±7)	374 (±33)	475 (±89)	959 (±154)	588 (±32)	627 (±50)	206 (±10)

* Values are means (±S.D.).

findings are consistent with changes in binding capacity resulting from changes in incubation temperature and Na^+ concentration.

Competition experiments showed marked differences in ligand binding profile between these two membrane preparations. In brain membranes, the binding sites labeled by [^{3}H]DHM (μ), [^{3}H]DADLE (δ) and [^{3}H]EKC ($\kappa + \mu$) show highest affinity to morphine, DADLE and EKC respectively (Table 7), while in cerebellar membranes μ sites have the same affinity to both morphine and DADLE, and δ sites show the same affinity to DADLE and morphine, or even higher affinity to morphine. EKC which should have high affinity to κ and μ sites also shows high affinity to the δ site. Since the relative content of the δ site is the same in brain and in cerebellum, the differences in apparent binding affinity (K_i) to prototype ligands suggest a preponderance of μ sites in rabbit cerebellum. Meunier [7] suggests that μ sites account for 80% of opiate receptor sites in rabbit cerebellum, on the basis of competition for [^{3}H]etorphine binding. However, sequential displacement of DIP by selective ligands indicates only 40% of μ sites.

The proportions of μ, δ, and κ sites found in rabbit cerebellum by sequential displacement were quite similar to those reported in rat brain [19, 23] while the proportions found in rabbit brain were close to those reported in guinea pig brain [24, 25].

From binding capacity data determined by saturation with labeled ligands or sequential displacement, β-EP sites appear to be approximately the sum of μ, δ and κ sites. This suggests that, β-EP can interact with all three receptors. However, saturation analysis gave a single K_d for β-EP which indicates that the β-EP binds to different sites with similar affinity. This conclusion is supported by the present findings that EKC, DADLE and morphine displaced [^{3}H]-β-EP with similar K_i values in brain and cerebellum (see Table 5) despite the large difference in receptor types composition.

After completing this manuscript, we noted the related publication of Meunier *et al.* [26]. Although different methods of analysis and different labeled compounds were used, both Meunier *et al.* [26] and we agree that there are approximately 3-fold higher levels of μ receptors in rabbit cerebellum than in brain, and approximately a 3-fold lower level of κ receptors in cerebellum than in brain. Our results indicate a substantial amount of δ receptors in both brain and cerebellum, while they indicate that the δ receptor is negligible. Further work will be required to clarify the status of δ receptors in rabbit cerebellum and brain.

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