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Multiple Opiate Binding Sites in the Central Nervous System of the Rabbit

Large Predominance of a *Mu* Subtype in the Cerebellum and Characterization of a *Kappa* Subtype in the Thalamus

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SUMMARY

We have compared the binding characteristics of [^{3}H]etorphine, a nonselective *mu*-, *delta*-, and *kappa*-opiate agonist, with those of [^{3}H]Tyr-D-Ala-Gly-MePhe-NH$(CH_2)_2$OH ([^{3}H]DAGO), a selective *mu*-agonist, in rabbit cerebellar and thalamic membranes. We have also examined the ability of various unlabeled opioid ligands to compete with the binding of [^{3}H]etorphine in the two preparations. In cerebellar membranes, [^{3}H]DAGO(K_d = 0.7 nM) labels slightly fewer sites than does [^{3}H]etorphine (K_d = 0.06 nM): 0.18 versus 0.24 pmole/mg of protein. In addition, competition studies indicate that up to 75% of the [^{3}H]etorphine binding sites in this preparation display (a) high apparent affinity for unlabeled DAGO and (b) higher apparent affinity for morphine, the prototypical *mu*-agonist, than for Tyr-D-Ala-Gly-Phe-D-Leu (DADL), a *delta*-agonist. Together, these results suggest that the rabbit cerebellum contains a very high proportion (0.7–0.8) of *mu*-opiate binding sites. In thalamic membranes, [^{3}H]DAGO (K_d = 1.1 nM) labels considerably fewer sites than does [^{3}H]etorphine (K_d = 0.08 nM): 0.09 versus 0.27 pmole/mg of protein. In this preparation, the competition curves of DAGO and of DADL resolve binding of [^{3}H]etorphine into two components. The first component accounts for 40–50% of total binding and reflects the interaction of [^{3}H]etorphine with *mu*-opiate binding sites. The second component (up to 50% of total binding) is unaffected in the presence of DADL at concentrations (1–10 μM) that rule out binding of [^{3}H]etorphine to *mu*- and *delta*-opiate binding sites. It disappears readily in the presence of very low concentrations (K_i < 1 nM) of benzomorphan opiates (bremazocine, cyclazocine, and ethylketocyclazocine) yet it is relatively insensitive to inhibition by *mu*- and *delta*-agonists. This second component may therefore reflect [^{3}H]etorphine's interaction with a *kappa*-opiate binding site. The *kappa*-opiate binding site is assayed for as that site which binds [^{3}H]etorphine (0.5 nM) in the presence of either DADL (2 μM) or 10 μM of another enkephalin: Tyr-D-Ser-Gly-Phe-Leu-Thr. We find that, in the rabbit central nervous system, the thalamus, followed by frontal cortex and caudate nucleus, shows the highest content of *kappa*-opiate binding sites.

INTRODUCTION

Early reports have demonstrated appreciable quantities of methionine-enkephalin (but not of leucine-enkephalin) (1) as well as high levels of [^{3}H]etorphine binding (2) in the rabbit cerebellum. Biochemical binding (2) and autoradiographic (3) studies have revealed that the [^{3}H]etorphine binding sites are present at a higher density in the molecular layer than in the granular layer of the cerebellar cortex. Since the cytoarchitecture (4) and physiology (5) of the cerebellum are particularly well documented, it seemed to us that this part of the rabbit central nervous system might well be the place to analyze the cellular and molecular events underlying normal action(s) of enkephalin.

However, the radioligand that we used in previous studies (2, 3) was [^{3}H]etorphine. Etorphine is nearly equipotent in inhibiting the electrically evoked contractions of the guinea pig ileum and of the mouse vas deferens (6) as well as in competing with the binding of opiates and of enkephalins in brain membranes (7, 8). In addition, [^{3}H]etorphine binds to a single component *in vitro*, and it is for this particular radioligand that brain

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membranes exhibit the highest binding capacity (9). Together, these results are generally taken to indicate that the oripavine interacts equally well with *mu* (8, 10), *delta* (8, 10), *kappa* (11), and benzomorphan (12) opiate receptor subtypes in nerve tissue. Therefore the subclassification of opiate receptor sites in the rabbit cerebellum remained to be determined.

In the present article, we have examined (a) the binding properties of [^{3}H]etorphine and those of [^{3}H]DAGO,[1] a *mu*-selective agonist with only low affinities for *delta* and *kappa* binding sites (13, 14), as well as (b) the ability of opioid ligands to compete with the binding of [^{3}H]etorphine in rabbit cerebellar and thalamic membranes. Thalamic membranes were chosen for comparison since, in rats, they show the highest (0.7) relative content of morphine (*mu*) receptors (12). This choice turned out to be of heuristic value, since it led us to identify a novel opiate binding site in rabbit thalamic membranes. Therefore, the present study, initially aimed at classifying opiate receptor subtypes in the rabbit cerebellum, was extended to the characterization and regional distribution of this novel site in the rabbit central nervous system.

We now report that (a) the rabbit cerebellum contains a fairly homogeneous (75%) population of *mu*-opiate binding sites. (b) In the rabbit thalamus, there are at least two classes of [^{3}H]etorphine binding sites in nearly equal amounts. One class binds [^{3}H]DAGO, a *mu*-agonist with high affinity, and may therefore represent *mu*-opiate binding sites. The other class displays very high apparent affinity for benzomorphan opiates yet a much lower apparent affinity for *mu*- and *delta*-agonists than does the *mu*-opiate binding site. It may therefore represent *kappa*-opiate binding sites. (c) In the rabbit central nervous system, the thalamus, followed by frontal cortex and caudate nucleus, shows the highest content of *kappa* binding sites.

MATERIALS AND METHODS

Young New Zealand White rabbits weighing 1.3–1.5 kg were obtained from a local farm. They were killed by decapitation. The following regions were rapidly dissected and weighed: frontal cortex, caudate nucleus, hippocampal formation, thalamus, mesencephalon, pons-medulla oblongata, cerebellum, and spinal cord (thoracolumbar).

Preparation of the CMF

The nerve tissue was homogenized at 4° in a total volume (v_0) of 1.2 ml/100 mg (wet weight) of 0.32 M sucrose in Tris-HCl, 1 mM (pH 7.4). Homogenization was completed in a Potter-Elvehjem tissue grinder by 10 strokes of a Teflon pestle, motor-driven at 800–1,000 rpm. The suspension was then incubated for 30 min at 35° to accelerate degradation of endogenous opioid peptides and centrifuged (0–2°) in a Beckman rotor (Type 30) for 30 min at 30,000 rpm. The pellet was resuspended (Polytron) in a large excess of ice-cold 50 mM Tris-HCl (pH 7.4) (hereafter referred to as "buffer") and centrifuged as before. The (washed) pellet was homogenized in v_0 of buffer to yield the CMF (4–6 ml of protein per milliliter).

[1] The abbreviations used are: DAGO, Tyr-D-Ala-Gly-MePhe-NH$(CH_2)_2$OH; CMF, crude membrane fraction; DADL, Tyr-D-Ala-Gly-Phe-D-Leu; DSTL, Tyr-D-Ser-Gly-Phe-Leu-Thr; EKC, ethylketocyclazocine; ES, enkephalin-sensitive [^{3}H]etorphine binding site; ER, enkephalin-resistant [^{3}H]etorphine binding site.

Direct Binding Studies and Competition Experiments in 50 mM Tris-HCl at pH 7.4

Direct binding studies. Each assay mixture (1.0 ml), in triplicate, contained 0.05 ml of CMF (0.2–0.3 mg of protein) and [^{3}H]etorphine or [^{3}H]DAGO at the desired concentration in the absence (total binding) and in the presence (nonspecific binding) of 10 μM levorphanol.

Competition experiments. Each assay mixture (1.0 ml) contained 0.05 ml of CMF and 0.5 nM of [^{3}H]etorphine (a) with no other addition (total binding, in sextuplate), (b) in the presence of the desired concentration of unlabeled ligand (in triplicate), and (c) in the presence of 10 μM levorphanol (nonspecific binding, in sextuplate).

After a 60-min incubation at 25°, the assays were cooled in melting ice, filtered on glass-fiber discs (Whatman GF/B), and washed with two 5-ml portions of ice-cold buffer. With the use of Millipore Model 1225 sampling manifolds, the entire procedure required about 5.5 min/48 samples. The filters were then dried and counted for radioactivity in 4 ml of Beckman Ready-Solv EP cocktail by a Kontron (Model MR 300) automatic liquid scintillation system.

Chemicals

[14,16-^{3}H]etorphine (46 and 34 Ci/mmole) and [3,5-^{3}H]Tyr-D-Ala-Gly-MePhe-NH$(CH_2)_2$OH (51 Ci/mmole) were purchased from Amersham International Ltd. (Amersham, England). DAGO was obtained from Cambridge Research Biochemicals Ltd. (Harston, England). DADL and Tyr-Pro-Phe-Pro-$CONH_2$ (morphiceptin) were generous gifts from Dr. H. Mazarguil (Toulouse). DSTL was kindly provided by Dr. B. Roques (Paris), as was bremazocine base by Sandoz (Basle). Morphine hydrochloride was obtained from Francopia (Paris), naloxone hydrochloride from Endo Laboratories (Garden City, N. Y.), cyclazocine base, and EKC methanesulfonate from Sterling Winthrop (Rensselaer, N. Y.).

RESULTS

Equilibrium binding of [^{3}H]etorphine and of [^{3}H]DAGO in cerebellar and thalamic membranes. Saturation experiments were carried out in rabbit cerebellar and thalamic membranes using [^{3}H]etorphine, a nonselective opiate agonist, and [^{3}H]DAGO, a highly *mu*-selective opioid peptide.

[^{3}H]Etorphine was found to bind with nearly the same high affinity (K_d = 0.06 nM in cerebellum, 0.08 nM in thalamus) to one single class of sites in the two preparations. Thalamic membranes exhibited a slightly higher capacity (B_{max} = 0.27 pmole/mg of protein) than did cerebellar membranes (B_{max} = 0.24 pmole/mg of protein) (Fig. 1).

With [^{3}H]DAGO, the situation was different. [^{3}H]-DAGO also bound, with the same high affinity (K_d = 0.7 nM in cerebellum, 1.1 nM in thalamus), to a homogeneous population of sites in the two preparations. However, at saturating concentration, [^{3}H]DAGO labeled considerably fewer sites in thalamic membranes (0.09 pmole/mg of protein) than it did in cerebellar membranes (K_d = 0.18 pmole/mg of protein (Fig. 1).

These differences were interpreted to indicate that a dramatically different proportion of *mu* binding sites existed in the two preparations: 75% in cerebellum, 33% in thalamus.

Inhibition of [^{3}H]etorphine binding by unlabeled opioid ligands in cerebellar and thalamic membranes. Since the equilibrium dissociation constants of [^{3}H]etorphine were identical (see above) in cerebellar and thalamic membranes, direct comparison could be made of

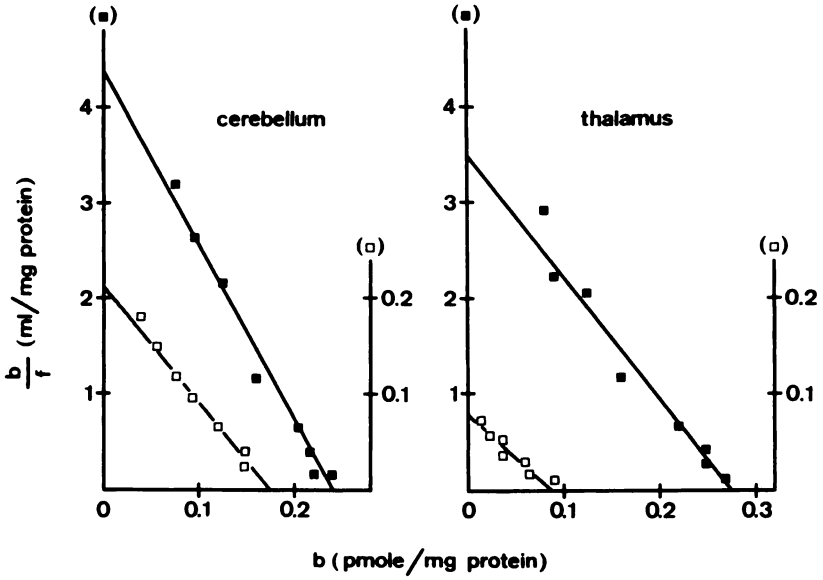

FIG. 1. *Saturation equilibrium binding of [^{3}H]etorphine (■) and of [^{3}H]DAGO (□) in rabbit cerebellar and thalamic membranes*
Scatchard representation. Each value is the mean of assays on four different preparations. Specific binding (*b*) was taken as the portion of total binding that was inhibited in the presence of 10 μM levorphanol. Note that, in comparison with [^{3}H]etorphine, which labels similar amounts of sites in the two preparations, [^{3}H]DAGO labels considerably fewer sites in thalamic membranes than it does in cerebellar membranes.

the ability of selected opioid ligands to compete with the binding of [^{3}H]etorphine used at the same fixed concentration (0.5 nM) in the two preparations.

In cerebellar membranes (Fig. 2), the binding competition curves of the *mu*-agonist, DAGO, and of the two *delta*-agonists, DADL and DSTL (15, 16), revealed a slight heterogeneity of [^{3}H]etorphine binding with a plateau corresponding to about 75% of inhibition. In contrast, the two other *mu*-agonists, morphine and morphiceptin (17); naloxone, an antagonist; and EKC, a benzomorphan opiate agonist, exerted apparent monotonic inhibition of [^{3}H]etorphine in this preparation. It is noteworthy that inhibition by morphiceptin was not biphasic, as one would have expected had sizable populations of *mu*- and *delta*-opiate binding sites been simultaneously present in cerebellar membranes. Here, the rank order of opiate inhibition of [^{3}H]etorphine binding was as follows: EKC ≈ naloxone ≈ DAGO > morphine > DADL > DSTL > morphiceptin. Again, these data suggest that the rabbit cerebellum contains a fairly homogeneous (75%) population of opiate binding sites which, because they display (a) high apparent affinity for DAGO and (b) higher apparent affinity for morphine than for DADL, are likely to represent *mu*-opiate binding sites.

In thalamic preparations, the situation was somewhat different. Naloxone and EKC excepted, all ligands produced clear biphasic inhibition of [^{3}H]etorphine binding, the two *delta*-agonists, DADL and DSTL, and the *mu*-agonist, DAGO, doing so in a more pronounced fashion than did the two other *mu*-agonists, morphine and morphiceptin (Fig. 3).

In fact, the use of the enkephalin in this test allowed clear distinction between at least two classes of [^{3}H] etorphine binding sites present in nearly equal amounts in the rabbit thalamus. For convenience, although somewhat improperly, these sites are hereafter referred to as enkephalin-sensitive (ES) and enkephalin-"resistant" (ER). Table 1 indicates that the concentrations of unlabeled opioid ligands causing 50% inhibition of [^{3}H]etorphine binding to the thalamic ES site are very close to the ones required to halve binding of [^{3}H]etorphine to the *mu*-opioid binding site in cerebellar membranes. The thalamic ES site may therefore represent a *mu*-opiate binding site.

Inhibition of [^{3}H]etorphine binding by unlabeled opioid ligands in the presence of 2 μM DADL in thalamic

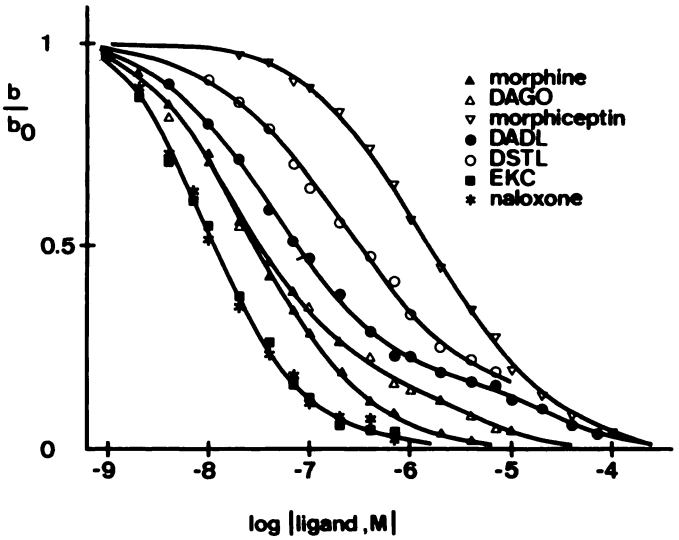

FIG. 2. *Inhibition of [^{3}H]etorphine binding by selected opioid ligands in rabbit cerebellar membranes*
[^{3}H]Etorphine was used at 0.5 nM. Each value is the mean of three assays (on different preparations) which were less than ±5% of the mean; *b* and b_0 represent specific [^{3}H]etorphine binding in the presence and in the absence of inhibitor, respectively.

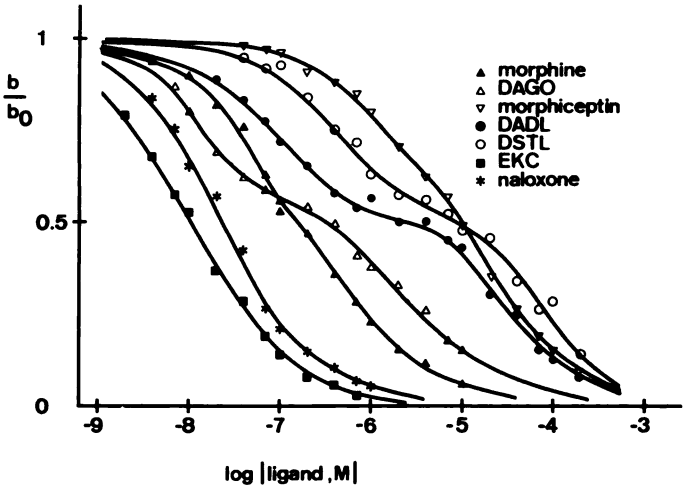

FIG. 3. *Inhibition of [^{3}H]etorphine binding by selected opioid ligands in rabbit thalamic membranes*

See legend to Fig. 2.

membranes. The thalamic ER site was further characterized by evaluating the potency of selected opioid ligands, including two additional benzomorphan opiates (cyclazocine and bremazocine) to inhibit binding of [^{3}H] etorphine in the presence of 2 μM DADL.

In the presence of 2 μM DADL, [^{3}H]etorphine still bound with very high affinity (K_d = 0.1 nM) but to a markedly reduced (50%) number of sites (Fig. 4) in the thalamic membranes. This "residual" binding could be attributed neither to *mu-* nor to *delta* sites, since 2 μM DADL represents >100 and >1000 times the dissociation constant of the pentapeptide for *mu-* and *delta*-opiate binding sites, respectively (16, 18).

Together with the data presented in Fig. 3, these data confirm the existence in the rabbit thalamus of at least two distinct classes of sites which bind etorphine equally well.

Under these conditions (presence of 2 μM DADL), the binding competition curves of all of the opioid ligands that we have tested appear to be monophasic as assessed by the linear Hill transformations, with slopes all close to unity (Fig. 5).

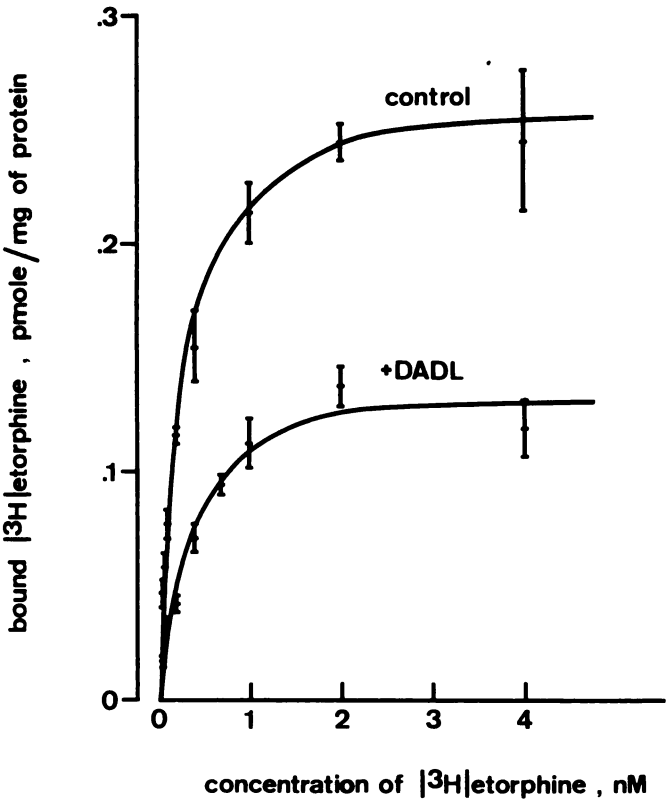

FIG. 4. *Saturation equilibrium binding of [^{3}H]etorphine in the absence and in the presence of 2 μM DADL in rabbit thalamic membranes*

Each value is the mean ± standard deviation (*vertical bars*) of triplicate assays on the same preparation. Specific binding (ordinate) was defined as in the legend to Fig. 1. Scatchard analysis of the data indicated identical affinities (K_d = 0.1 nM) in the two cases (± DADL), yet, in the presence of 2 μM DADL, [^{3}H]etorphine interacted with a markedly reduced (50%) number of binding sites.

The salient property of the ER site resides in its extremely high apparent affinity for benzomorphan opiates (K_i < 1 nM) and in its several orders of magnitude lower apparent affinity for *mu-* and for *delta*-agonists (Table 2). These findings suggest that the thalamic ER site actually represents a *kappa*-opiate binding site.

TABLE 1

Potency of selected opioid ligands to compete with the binding of [^{3}H]etorphine in cerebellar and thalamic membranes

[^{3}H]Etorphine was used at 0.5 nM. I_{25}, I_{50}, and I_{75} are the concentrations of unlabeled ligand causing 25%, 50%, and 75% inhibition, respectively. The values in parentheses represent I_{50} values of EKC and of naloxone, which exerted apparent monotonic inhibition of [^{3}H]etorphine binding in thalamic membranes.

Ligand	Cerebellum, I_{50}	Thalamus		I_{25} (thalamus)/ I_{50} (cerebellum)	I_{75} (thalamus)/ I_{25} (thalamus)
		I_{25}	I_{75}		
	nM	nM			
Mu-agonists					
Morphine	28	38	830	1.4	22
DAGO	15	11	2,500	0.7	227
Morphiceptin	1,500	1,500	40,000	1.0	27
Delta-agonists					
DADL	42	76	35,000	1.8	460
DSTL	170	400	79,000	2.4	198
Kappa-agonist					
EKC	11	— (11)	—	—	—
Antagonist					
Naloxone	11	— (23)	—	—	—

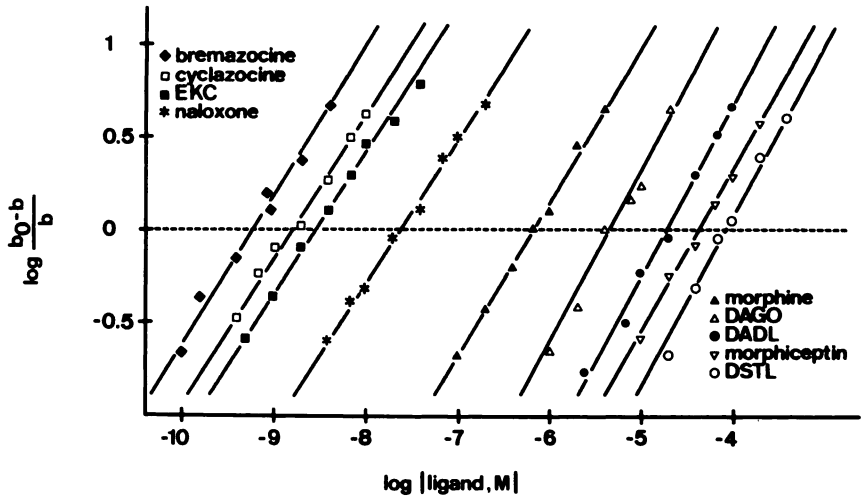

FIG. 5. *Inhibition of [^{3}H]etorphine binding by selected opioid ligands in the presence of 2 μM DADL in rabbit thalamic membranes*

[^{3}H]Etorphine was used at 0.5 nM. To improve, under these conditions (presence of 2 μM DADL), identification of the [^{3}H]etorphine binding site as a *kappa* site, two additional benzomorphan opiates were tested: bremazocine and cyclazocine. Each value is the mean of three determinations (on different preparations) which were less than ±5% of the mean; b and b_0 are defined in the legend to Fig. 2. Hill transformation.

TABLE 2

Potency of selected ligands to compete with the binding of [^{3}H] etorphine (0.5 nM) in the presence of 2 μM DADL in rabbit thalamic membranes

K_i values were calculated assuming competitive inhibition as follows: $K_i = I_{50}/(1 + L/K_d)$, where L is the concentration of free radioligand and K_d is the equilibrium dissociation constant of the radioligand. I_{50} values were determined graphically from the modified Hill transformation in Fig. 5.

Ligand	I_{50}	K_i
	nM	*nM*
Mu-agonists		
Morphine	710	180
DAGO	4,800	1,250
Morphiceptin	48,000	12,500
Delta-agonists		
DADL	19,000	4,000
DSTL	83,000	21,600
Kappa-agonists		
EKC	3.0	0.80
Cyclazocine	2.9	0.75
Bremazocine	0.6	0.16
Antagonist		
Naloxone	22	5.7

Regional distribution of the kappa-opiate binding site in the rabbit central nervous system. The *kappa*-opiate binding site was assayed for in membrane suspensions from various regions of the rabbit central nervous system as that site which binds [^{3}H]etorphine (0.5 nM) in the presence of either DADL (2 μM) or of 10 μM of DSTL.

Table 3 indicates that, in the absence of enkephalin, binding of the tritiated oripavine was highest in caudate nucleus, followed by frontal cortex, and lowest in hippocampal formation and spinal cord. In the presence of either of the two enkephalins, DADL or DSTL, it was the thalamus, followed by frontal cortex, that showed the highest levels of [^{3}H]etorphine binding. However, when expressed in terms of relative content of *kappa*-opiate binding sites, the data indicated the following rank order: thalamus > spinal cord > frontal cortex > pons-medulla oblongata ≈ caudate nucleus ≈ mesencephalon ≈ hippocampal formation > cerebellum.

DISCUSSION

Our data point to the existence of at least two distinct classes of opiate binding sites in rabbit cerebellar and thalamic membranes. These two classes, referred to, for

TABLE 3

Regional distribution of (mu + delta + kappa) and of kappa-binding sites in the central nervous system of the rabbit

Crude membranes were prepared from the various regions as described under Materials and Methods. *Mu*, *delta*, and *kappa* binding sites were assayed as a whole with 0.5 nM [^{3}H]etorphine (control). *Kappa* binding sites were assayed for as those sites which bind [^{3}H]etorphine (0.5 nM) in the presence of either 2 μM DADL or of 10 μM DSTL; n is the number of independent assays.

Region	Protein (n = 6)	Bound [^{3}H]etorphine			% Control	
		Control (n = 6)	+DADL (n = 3)	+DSTL (n = 3)	+DADL	+DSTL
	mg/ml	*fmoles/mg protein*				
Frontal cortex	0.23 ± 0.03	213 ± 16	58 ± 5	70 ± 10	28 ± 4	33 ± 2
Caudate nucleus	0.20 ± 0.03	239 ± 23	53 ± 3	46 ± 2	21 ± 1	21 ± 1
Hippocampal formation	0.21 ± 0.03	71 ± 8	10 ± 5	15 ± 4	15 ± 8	21 ± 4
Thalamus	0.30 ± 0.04	177 ± 15	81 ± 4	82 ± 5	47 ± 3	48 ± 2
Mesencephalon	0.22 ± 0.02	151 ± 14	30 ± 6	33 ± 5	19 ± 5	23 ± 3
Pons-medulla oblongata	0.25 ± 0.02	91 ± 5	21 ± 2	21 ± 3	22 ± 2	24 ± 3
Cerebellum	0.25 ± 0.03	195 ± 15	32 ± 5	29 ± 3	15 ± 1	16 ± 1
Spinal cord	0.17 ± 0.02	32 ± 6	14 ± 6	12 ± 1	39 ± 9	42 ± 4

convenience, as ES and ER, appear to represent *mu* and *kappa* binding sites, respectively.

Mu binding sites in rabbit cerebellar and thalamic membranes. A *mu*-opiate binding site was identified in the two preparations on the following bases: (a) It binds [^{3}H]DAGO, a highly *mu*-selective radioligand, with the same high affinity ($K_A = 1$ nM^{-1}) as that reported for the [^{3}H]dihydromorphine (*mu*) binding site in guinea pig brain membranes (14). (b) It exhibits lower (yet high) apparent affinity for DADL, a *delta*-agonist, than it does for morphine, a *mu*-agonist (8). (c) Its apparent affinities for morphine, DADL, EKC, and naloxone are virtually identical with those of the [^{3}H]dihydromorphine (*mu*) binding site in guinea pig brain membranes (11).

Our saturation and competition studies have yielded consistent data and have shown that rabbit *cerebellar* membranes contain a very high proportion (75%) of *mu*-opiate binding sites. This result may bear interesting theoretical and practical implications. For instance, one knows that the rabbit cerebellum contains appreciable quantities of methionine-enkephalin but virtually no leucine-enkephalin (1). It is therefore tempting to infer that methionine-enkephalin normally acts as a physiological agonist at *mu* "receptors" in this organ, and possibly in the rest of the central nervous system. This hypothesis was first put forward by Snyder and Goodman (19) on the basis of binding and autoradiographic studies.

Recently, enkephalin-like immunoreactive Golgi neurons (20, 21) and mossy fibers (21) have been detected in cerebellum of several species, rabbit included. Golgi neurons and mossy fibers have long been known to terminate in the granular layer of the cerebellar cortex (22) Assuming that it is in this layer that enkephalins are released, our previous observation that [^{3}H]etorphine binding sites predominate in the molecular layer (2, 3) raises the possibility that enkephalin normally acts at long distance, i.e., as neurohormones in the rabbit cerebellum.

In practice, rabbit cerebellar membranes may also facilitate the physicochemical characterization of a *mu*-opiate "receptor"[2] regardless of ligand cross-reactivity (18). In thalamic membranes, the proportion of *mu*-opiate binding sites was found to be slightly different in [^{3}H]DAGO saturation studies (33%) and in [^{3}H]etorphine binding competition studies (up to 50%). This small quantitative discrepancy may be attributed to the existence of a correspondingly small proportion of *delta*-opiate binding sites in this preparation. Along this line, it should be noted that Chang *et al.* (12) have reported an extremely low relative content of *delta*-opiate binding sites in rat thalamic membranes. As far as *mu*-binding sites are concerned, it is particularly striking that their proportion varies so much in the same region of the brains of the different species: 70% in rat thalamus (12) versus 33% in rabbit thalamus (present study).

Kappa binding sites in the rabbit central nervous system. A *kappa*-opiate binding site was identified in thalamic membranes on the following bases: (a) It binds [^{3}H]etorphine with very high affinity ($K_A = 10$ nM^{-1}) in the presence of 2 μM DADL, i.e., under experimental conditions that preclude any interaction of the tritiated oripavine with *mu*- and *delta*-opiate binding sites. (b) It exhibits several orders of magnitude higher apparent affinity for the benzomorphan opiates bremazocine, cyclazocine, and EKC than it does for *mu*- and for *delta*-agonists (see Table 2). (c) Its apparent affinities for morphine, DAGO, DADL, bremazocine, EKC, and naloxone are comparable to those of the [^{3}H]EKC (*kappa*) binding site in guinea pig brain membranes (11).

Recently, Chang *et al.* (12) have presented evidence for a novel opiate "receptor" site selective for benzomorphan drugs in rat brain membranes. Although this site was characterized in much the same way as was our *kappa* site (Chang *et al.* used [^{3}H]diprenorphine instead of [^{3}H]etorphine), the two sites (benzomorphan and *kappa*) appear to be different. In particular, benzomorphan and *kappa* sites display, respectively, 30-fold and >6000-fold higher apparent affinities for EKC than they do for DADL. Moreover, the regional distribution of the benzomorphan site was reported to resemble closely that of the *mu* site in the rat brain. This is certainly not the case for *mu* and *kappa* sites in the rabbit cerebellum and thalamus.

In conclusion, (a) the *kappa* binding site which we have characterized in the rabbit thalamus is closely related to, if not identical with, the *kappa* binding site described by Kosterlitz *et al.* (11) in guinea pig membranes. It is not known whether or not the *kappa* site is similarly distributed in the two species, since its regional distribution in the central nervous system of the guinea pig has not yet been reported. (b) The *kappa* binding site is different from the benzomorphan site described by Chang *et al.* (12) in rat brain membrane. This difference may actually reflect a duality of *kappa* binding sites, as it has been reported in peripheral tissue (23) and in human brain (24).

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