[³H]-β-ENDORPHIN BINDING IN RAT BRAIN¹

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Abstract

The binding of [³H]-β-endorphin to rat brain homogenates is complex. Although Scatchard analysis of saturation studies yields a straight line, detailed competition studies are multiphasic, suggesting that even at low concentrations of the compound, the ³H-ligand is binding to more than one class of site. A portion of [³H]-β-endorphin binding is sensitive to low concentrations of morphine or D-Ala²-Leu⁵-enkephalin (<5 nM). The inhibition observed with each compound alone (5 nM) is the same as that seen with both together (each at 5 nM). Thus, the binding remaining in the presence of both morphine and the enkephalin does not correspond to either μ or δ sites. The portion of [³H]-β-endorphin binding that is inhibited under these conditions appears to be equally sensitive to both morphine and the enkephalin and may correspond to μ₁ sites. Treating membrane homogenates with naloxonazine, a μ₁ selective antagonist, lowers [³H]-β-endorphin binding to the same degree as morphine and D-Ala²-Leu⁵-enkephalin alone or together. This possible binding of [³H]-β-endorphin to μ₁ sites is consistent with the role of μ₁ sites in β-endorphin analgesia and catalepsy *in vivo*.

The role of subpopulations of opioid receptors has become increasingly important in our understanding of opioid action. Extensive studies in the chronic spinal dog led to the suggestion of three classes of alkaloid receptors, named after prototypic compounds: μ (morphine), κ (ketocyclazocine), and σ (SKF 10,047; Martin et al., 1976). Soon after the discovery of the enkephalins, Lord et al. (1977) demonstrated marked differences between morphine and the enkephalins in the guinea pig ileum and mouse vas deferens bioassays, leading to the hypothesis of discrete enkephalin-preferring (δ) receptors. In a similar approach, the presence of receptors selective for β-endorphin (ε) has been suggested in studies involving the rat vas deferens bioassay (Wuster et al., 1980; Schulz et al., 1981). Receptor binding studies have also demonstrated differences between the binding of radiolabeled β-endorphin and other opiates and opioid peptides (Ferrara et al., 1979; Hazum et al., 1979; Law et al., 1979; Akil et al., 1980, 1981; Ferrara and Li, 1980a, b; Houghten et al., 1980; Hammonds and Li, 1981, 1982; Johnson et al., 1982; Ho et al., 1983; Lin-Shiau et al., 1983). In addition, autoradiography of [³H]-β-endorphin binding in rat brain also shows regional differences from [³H]dihydromorphine and [³H]-D-Ala²-D-Leu⁵-enkephalin binding, suggestive of β-endorphin-selective sites (Goodman et al., 1983). *In vivo* studies have

implied a role of μ₁ sites in β-endorphin analgesia and catalepsy (Pasternak, 1981; Ling and Pasternak, 1982). We now present further studies on [³H]-β-endorphin binding confirming the existence of distinct β-endorphin-binding sites which differ from classical μ and δ sites and demonstrating for the first time the binding of [³H]-β-endorphin to sites which appear to correspond to μ₁ sites.

Materials and Methods

[³H]-β-endorphin (48 Ci/mmol) was synthesized as previously reported and verified as authentic β-endorphin by high pressure liquid chromatography (HPLC) (Houghten and Li, 1978). [³H]Dihydromorphine (70 Ci/mmol), [³H]-D-Ala²-D-Leu⁵-enkephalin (38 Ci/mmol), [³H]-(±)-ethylketocyclazocine (16.4 Ci/mmol), [³H]-(±)-SKF 10,047 (30.7 Ci/mmol), and Formula 973 scintillation fluor were purchased from New England Nuclear Corp. (Boston, MA). Unlabeled D-Ala²-Leu⁵-enkephalin and β-endorphin were obtained from Beckman (Palo Alto, CA). Morphine, (±)-ethylketocyclazocine, and (±)-SKF 10,047 (*N*-allylnormetazocine) were obtained from the National Institute of Drug Abuse. Naloxonazine was synthesized as previously described (Hahn et al., 1982). Male Sprague-Dawley rats (180 gm; Crl:CD(SD)BD) were purchased from Charles River Breeding Laboratories. All peptides were synthesized by solid phase methods (Houghten et al., 1980).

Binding assays were performed at 25°C as previously described (Pasternak et al., 1975) with certain modifications. All incubations with radiolabeled drugs were carried out for 60 min to ensure reaching equilibrium (Johnson et al., 1982). Tris buffer (pH 7.4 at 25°C) was used since this corresponded to the pH optimum for binding (R. A. Houghten, unpublished observations). Bacitracin (50 μg/ml) was added to the tissue for all assays using radiolabeled or unlabeled β-endorphin to minimize radioligand degradation. Previous studies have shown that under these conditions, less than 5% of β-endorphin is degraded as determined by carboxymethylcellulose chromatography and HPLC. Only plastic tubes were used with assays involving radiolabeled or

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unlabeled $\beta\text{-endorphin}$ to minimize the binding of the ligand to the tube walls. Nonspecific binding to glass fiber filters was minimized by treating the filters with polyethylenimine (0.1%) prior to filtering the tissue (R. A. Houghten, unpublished observations). Nonspecific binding was determined in all assays as the binding remaining in the presence of levallorphan (1 μM). All $[^3\text{H}]\text{-}\beta\text{-endorphin}$ binding displaced by unlabeled $\beta\text{-endorphin}$ at concentrations up to 100 nM was also displaced by levallorphan (1 μM) and routinely consisted of about 65% of total binding. All experiments were performed at least three times with

similar results. Naloxonazine (50 nM) treatment was performed at 25°C for 30 min, and the tissue was then centrifuged ($49,000 \times g$ for 20 min), resuspended, and washed twice, as previously described (Hahn et al., 1982). Each wash consisted of an incubation at 37°C for 10 min followed by another centrifugation and resuspension. This procedure effectively removes the inhibition of binding by reversible opiates (Hahn et al., 1982). Estimations of K_D and B_{max} values were made by nonlinear regression analysis of the saturation data.

Results

Characterization of $[^3\text{H}]\text{-}\beta\text{-endorphin}$ binding. Scatchard analysis of saturation studies of $[^3\text{H}]\text{-}\beta\text{-endorphin}$ binding (Fig. 1) demonstrates a high affinity binding component with a K_D value of 0.6 nM and a B_{max} of 6.7 fmol/mg of tissue. Similar results have been obtained in a variety of other studies. This binding is dependent upon tissue. The mean $[^3\text{H}]\text{-}\beta\text{-endorphin}$ binding in three separate filtration assays (5294 ± 340 cpm) is virtually identical to that observed in three separate microfuge centrifugation assays (4821 ± 980 cpm). In addition, $[^3\text{H}]\text{-}\beta\text{-endorphin}$ binding is biochemically similar to $[^3\text{H}]\text{-dihydromorphine}$ and $\text{D-Ala}^2\text{-D-Leu}^5\text{-enkephalin}$ binding, being highly dependent upon the integrity of membrane proteins and sulfhydryl groups and showing the same sensitivity to sodium ions as other opioid agonists (Table I).

Pharmacological specificity of $[^3\text{H}]\text{-}\beta\text{-endorphin}$ binding. The binding of $[^3\text{H}]\text{-}\beta\text{-endorphin}$ is opioid in nature. Both morphine and $\text{D-Ala}^2\text{-Leu}^5\text{-enkephalin}$ inhibit $[^3\text{H}]\text{-}\beta\text{-endorphin}$ binding, but with IC_{50} values 2-fold and 3-fold higher, respectively, than unlabeled $\beta\text{-endorphin}$ (Table II). Various other opioid peptides also compete for $[^3\text{H}]\text{-}\beta\text{-endorphin}$ binding (Table III). However, the sensitivity of $[^3\text{H}]\text{-}\beta\text{-endorphin}$ binding for these peptides differs from that of the other ^3H -opioids. For example, the potent δ peptide $\text{D-Ala}^2\text{-Met}^5\text{-enkephalin}$ inhibits $[^3\text{H}]\text{-D-}$

Figure 1. Saturation studies of $[^3\text{H}]\text{-}\beta\text{-endorphin}$ binding. Rat brain homogenates were prepared as described under "Materials and Methods," and binding was performed with $[^3\text{H}]\text{-}\beta\text{-endorphin}$ (0.05 to 1.3 nM). Points represent the means $\pm$ SEM of triplicate samples from a representative experiment. The experiment has been replicated three times with similar results.

TABLE I

Effect of sodium chloride, N-ethylmaleimide, and trypsin on ^3H -opioid binding

Rat brain homogenates were prepared and incubated with either nothing, N-ethylmaleimide (25 μM), or trypsin (1 $\mu\text{g}/\text{ml}$) for 30 min at 25°C, centrifuged, and assayed with $[^3\text{H}]\text{-}\beta\text{-endorphin}$, $[^3\text{H}]\text{-dihydromorphine}$, or $[^3\text{H}]\text{-D-Ala}^2\text{-D-Leu}^5\text{-enkephalin}$ (all at 0.75 nM). Untreated tissue was also assayed in the presence and absence of NaCl (50 mM). Results are the mean $\pm$ SEM of the inhibitions from three separate experiments. Differences between the three ^3H -ligands were determined for each treatment by analysis of variance: NaCl, $F_{2,6} = 0.15$, NS; N-ethylmaleimide, $F_{2,6} = 1.45$, NS; trypsin, $F_{2,6} = 11.03$, $p < 0.01$.

Treatment	Inhibition of Binding			
	$[^3\text{H}]\text{-}\beta\text{-Endorphin}$	$[^3\text{H}]\text{-Dihydromorphine}$	$[^3\text{H}]\text{-D-Ala}^2\text{-D-Leu}^5\text{-enkephalin}$	
	%	%	%	
NaCl	70.0 ± 2.9	65.0 ± 10.6	68.0 ± 2	NS ^a
N-Ethylmaleimide	28.3 ± 7.8	41.7 ± 15	53.3 ± 6	NS
Trypsin	88.7 ± 1.9	54.7 ± 5.9	61.0 ± 7	$p < 0.01$

^a NS, not significant.

TABLE II

Inhibition of ^3H -opioid binding by $\beta\text{-endorphin}$ and by combinations of morphine and $\text{D-Ala}^2\text{-Leu}^5\text{-enkephalin}$

Homogenates were prepared and assays were performed as described under "Materials and Methods" with each ^3H -ligand at 0.75 nM. Each value is the mean $\text{IC}_{50} \pm$ SEM of n separate determinations. The values of morphine + $\text{D-Ala}^2\text{-Leu}^5\text{-enkephalin}$ (5 nM) denote the IC_{50} of morphine determined in the presence of a fixed concentration (5 nM) of the enkephalin. Likewise, the values for $\text{D-Ala}^2\text{-Leu}^5\text{-enkephalin}$ + morphine (5 nM) represent the IC_{50} values of $\text{D-Ala}^2\text{-Leu}^5\text{-enkephalin}$ determined in the presence of a fixed concentration of morphine (5 nM). Values in parentheses represent the number of determinations.

Compound	IC_{50} Values		
	$[^3\text{H}]\text{-}\beta\text{-Endorphin}$	$[^3\text{H}]\text{-Dihydromorphine}$	$[^3\text{H}]\text{-D-Ala}^2\text{-D-Leu}^5\text{-enkephalin}$
		nM	
$\beta\text{-Endorphin}$	4.7 ± 0.9 (3)	5.3 ± 0.6 (3)	5.1 ± 0.4 (6)
Morphine	9.1 ± 0.8 (7)	3.2 ± 0.7 (5)	
+ $\text{D-Ala}^2\text{-Leu}^5\text{-enkephalin}$ (5 nM)	11 ± 2 (3)	2.3 ± 0.4 (4)	
$\text{D-Ala}^2\text{-Leu}^5\text{-enkephalin}$	14 ± 3 (5)		3.5 ± 0.3 (8)
+ Morphine (5 nM)	23 ± 5 (3)		2.1 ± 0.3 (3)

TABLE III
Inhibition of ^3H -opioid binding by opioid peptides

Rat brain homogenates were prepared and assayed with the stated ^3H -opioid (0.75 nM) and the various opioid peptides at 25°C as described under "Materials and Methods." Results are mean $\pm$ SEM of n separate experiments expressed as inhibition of specific binding. Values in parentheses represent the number of determinations.

	Inhibition of Binding			
	$[\text{H}]\text{-}\beta\text{-Endorphin}$	$[\text{H}]\text{-D-Ala}^2\text{-D-Leu}^5\text{-enkephalin}$	$[\text{H}]\text{-Dihydro-morphine}$	$[\text{H}]\text{-Ethylketocyclazocine}$
	%	%	%	%
Tyr-Gly (5 nM)	21 $\pm$ 11 (5)	24 $\pm$ 6 (4)	16 $\pm$ 5 (4)	13 $\pm$ 7 (4)
D-Ala ² -Leu ⁵ -enkephalin (5 nM)	35 $\pm$ 3 (5)	63 $\pm$ 6 (4)	50 $\pm$ 6 (4)	51 $\pm$ 6 (4)
D-Ala ² -Met ⁵ -enkephalin (5 nM)	19 $\pm$ 3 (5)	75 $\pm$ 6 (4)	23 $\pm$ 4 (4)	30 $\pm$ 7 (4)
D-Ala ² -N-MePhe ⁴ -Met(O)-Ox ⁵ -enkephalin (FK33824)	49 $\pm$ 2 (5)	42 $\pm$ 2 (3)	75 $\pm$ 3 (5)	58 $\pm$ 7 (4)
D-Ala ² -Met ⁵ -enkephalinamide (5 nM)	53 $\pm$ 3 (5)	63 $\pm$ 11 (3)	52 $\pm$ 7 (4)	42 $\pm$ 12 (3)
Dynorphin-A (20 nM)	55 $\pm$ 4 (3)	36 $\pm$ 12 (3)	73 $\pm$ 4 (2)	72 $\pm$ 1 (2)
Dynorphin-A (1-8) (20 nM)	22 $\pm$ 11 (3)	17 $\pm$ 14 (2)	20 $\pm$ 4 (2)	7 $\pm$ 6 (2)
Dynorphin-A (1-13) (20 nM)	46 $\pm$ 9 (3)	35 $\pm$ 1 (3)	45 $\pm$ 3 (2)	51 $\pm$ 2 (2)
Dynorphin-A (10-17) (20 nM)	38 $\pm$ 12 (3)	46 $\pm$ 13 (3)	43 $\pm$ 8 (2)	19 $\pm$ 7 (2)
Dynorphin-B (1-13) (20 nM)	11 $\pm$ 3 (3)	16 $\pm$ 3 (3)	18 $\pm$ 10 (2)	29 $\pm$ 3 (2)
α -Neoendorphin (20 nM)	28 $\pm$ 1 (3)	32 $\pm$ 5 (3)	43 $\pm$ 2 (2)	36 $\pm$ 2 (2)

Ala²-D-Leu⁵-enkephalin binding by 75% while decreasing $[\text{H}]\text{-}\beta\text{-endorphin}$ binding only 19%. On the other hand, the potent μ peptide FK33,824 decreases $[\text{H}]\text{-dihydromorphine}$ binding by 75% compared to only 49% of $[\text{H}]\text{-}\beta\text{-endorphin}$. Similarly, $[\text{H}]\text{-}\beta\text{-endorphin}$ binding is less sensitive to the dynorphin peptides as well as α -neoendorphin.

All of the $[\text{H}]\text{-}\beta\text{-endorphin}$ binding displaced by unlabeled $\beta\text{-endorphin}$ is also competed by other opioids but with lower potency (Fig. 2). Although the Scatchard plot of $[\text{H}]\text{-}\beta\text{-endorphin}$ binding is linear over the concentration range used in our experiments, the competition curves suggest that $[\text{H}]\text{-}\beta\text{-endorphin}$ is binding to more than one site. Approximately 20 to 35% of $[\text{H}]\text{-}\beta\text{-endorphin}$ binding is potently displaced by low concentrations of morphine, D-Ala²-Leu⁵-enkephalin, ethylketocyclazocine, and SKF 10,047 with a potency similar to that of $\beta\text{-endorphin}$ itself. Unlabeled $\beta\text{-endorphin}$ displaces the remainder of the $[\text{H}]\text{-}\beta\text{-endorphin}$ binding more effectively than the other opioids, consistent with the possibility of $\beta\text{-endorphin}$ -selective sites. However, other possibilities must be considered.

It has been suggested that $\beta\text{-endorphin}$ binding might consist of the labeling of both μ and δ sites (Lee and Smith, 1980). Clearly, $\beta\text{-endorphin}$ will bind to μ and δ sites, illustrated by its ability to inhibit both $[\text{H}]\text{-dihydromorphine}$ and D-Ala²-D-Leu⁵-enkephalin binding (Table II). In this model, morphine alone would effectively inhibit binding to μ sites, leaving the binding of $[\text{H}]\text{-}\beta\text{-endorphin}$ to δ sites, for which morphine has poor affinity. A similar situation would exist with enkephalin competition studies. One approach to test this hypothesis is to examine the competition of $[\text{H}]\text{-}\beta\text{-endorphin}$ binding by each compound in the absence and presence of a fixed concentration of the other. If $[\text{H}]\text{-}\beta\text{-endorphin}$ binding merely consisted of a combination of μ and δ binding, the μ binding of $[\text{H}]\text{-}\beta\text{-endorphin}$ could be competed with a fixed concentration of morphine, leaving the binding of $[\text{H}]\text{-}\beta\text{-endorphin}$ to δ sites. Under these conditions, the remaining $[\text{H}]\text{-}\beta\text{-endorphin}$ binding should become quite sensitive to enkephalins. D-Ala²-Leu⁵-enkephalin inhibits $[\text{H}]\text{-}\beta\text{-endorphin}$ binding with an IC_{50} value of 14 nM as opposed to its IC_{50} value against $[\text{H}]\text{-D-Ala}^2\text{-D-Leu}^5\text{-enkephalin}$ of 3.5 nM. In the presence of morphine the enkephalin inhibits $[\text{H}]\text{-}\beta\text{-endorphin}$ binding with an IC_{50} value of 23 nM, as opposed to its IC_{50} value of 2.3 nM against D-Ala²-D-Leu⁵-enkephalin. Similarly, the IC_{50} of morphine against $[\text{H}]\text{-}\beta\text{-endorphin}$ in the presence of the enkephalin is 11 nM, a value more than 4-fold higher than morphine's IC_{50} against $[\text{H}]\text{-dihydromorphine}$ in the presence of the enkephalin (2.3 nM).

Figure 2. Displacement studies of $[\text{H}]\text{-}\beta\text{-endorphin}$ binding. Rat brain homogenates were prepared as described under "Materials and Methods," and binding was performed with $[\text{H}]\text{-}\beta\text{-endorphin}$ at 0.75 nM. A, Displacements were performed with unlabeled $\beta\text{-endorphin}$ (solid circles), morphine (open circles), and D-Ala²-D-Leu⁵-enkephalin (open squares). B, Displacements were performed with ethylketocyclazocine (open triangles) and SKF 10,047 (solid squares). All experiments were performed at least three times. Points represent the means $\pm$ SEM of triplicate determinations from a representative experiment.

Similar findings are observed in the full competition studies of morphine on $[\text{H}]\text{-}\beta\text{-endorphin}$ binding in the presence and absence of a fixed concentration of D-Ala²-Leu⁵-enkephalin and with D-Ala²-Leu⁵-enkephalin in the presence and absence of a fixed concentration of morphine (Fig. 3). $[\text{H}]\text{-}\beta\text{-endorphin}$ binding is displaced in a biphasic manner by both morphine and D-Ala²-Leu⁵-enkephalin. The addition of either unlabeled compound alone at 5 nM displaces approximately 50% of spe-

Figure 3. Displacement of $[^3\text{H}]\text{-}\beta\text{-endorphin}$ binding by morphine and D-Ala²-Leu⁵-enkephalin alone and together. Rat brain homogenates were prepared as described under "Materials and Methods," and binding was performed with $[^3\text{H}]\text{-}\beta\text{-endorphin}$ (0.75 nM). *Top*, Binding was displaced with varying concentrations of morphine sulfate in the absence (solid circles) and presence of a fixed concentration of D-Ala²-Leu⁵-enkephalin (5 nM; open circles). *Bottom*, Binding was displaced with varying concentrations of D-Ala²-Leu⁵-enkephalin in the absence (solid circles) and presence of a fixed concentration of morphine sulfate (5 nM; open circles). Results are the means $\pm$ SEM of triplicate samples from a representative experiment. Both experiments have been replicated with similar results three times.

cific $[^3\text{H}]\text{-}\beta\text{-endorphin}$ binding. However, this ability of morphine at concentrations up to 5 nM to lower $[^3\text{H}]\text{-}\beta\text{-endorphin}$ binding is almost totally lost when the assay is performed in the presence of D-Ala²-Leu⁵-enkephalin (5 nM). Similarly, the inclusion of morphine at 5 nM eliminates any additional inhibition of $[^3\text{H}]\text{-}\beta\text{-endorphin}$ binding by concentrations of D-Ala²-Leu⁵-enkephalin up to 5 nM. Thus, the portion of $[^3\text{H}]\text{-}\beta\text{-endorphin}$ binding sensitive to low concentrations of morphine is also very sensitive to low concentrations of D-Ala²-Leu⁵-enkephalin.

We next performed saturation studies with $[^3\text{H}]\text{-}\beta\text{-endorphin}$ alone, in the presence of either morphine or enkephalin alone, or both compounds together (Fig. 4). At concentrations of 10 nM, more than 10-fold greater than their IC_{50} values for the initial displacements observed in the competition studies, both morphine and enkephalin alone decrease $[^3\text{H}]\text{-}\beta\text{-endorphin}$ binding by approximately 40%. The inclusion of both drugs together (each at 5 nM) inhibits $[^3\text{H}]\text{-}\beta\text{-endorphin}$ binding by 50%, a value very similar to that seen with either compound alone. These findings are consistent with the proposal that both compounds are competing with $[^3\text{H}]\text{-}\beta\text{-endorphin}$ for the same site and imply that the $[^3\text{H}]\text{-}\beta\text{-endorphin}$ binding remaining in the presence of morphine and D-Ala²-Leu⁵-enkephalin does not correspond to either μ or δ sites.

Effects of naloxonazine on $[^3\text{H}]\text{-}\beta\text{-endorphin}$ binding. Previous studies *in vivo* have suggested a role of μ_1 sites in $\beta\text{-endorphin}$'s analgesic and cataleptic actions (Pasternak, 1981; Zhang and Pasternak, 1981; Ling and Pasternak, 1982). The suggestion from the competition studies that a portion of $[^3\text{H}]\text{-}\beta\text{-endorphin}$ binding is potently displaced by both mor-

phine and D-Ala²-Leu⁵-enkephalin raises the question of whether this binding component might correspond to μ_1 sites. Naloxonazine is a potent, long-lasting antagonist selective for μ_1 sites. Treating tissue with naloxonazine (50 nM) followed by extensive washing lowers $[^3\text{H}]\text{-}\beta\text{-endorphin}$ binding approximately 40% (Table IV), a value similar to the inhibition of

Figure 4. Saturation studies of $[^3\text{H}]\text{-}\beta\text{-endorphin}$ in the absence and presence of opioids. *A*, Rat brain homogenates were prepared and binding was performed with $[^3\text{H}]\text{-}\beta\text{-endorphin}$ (0.06 to 1.6 nM) in the absence (solid circles) and presence of either morphine (10 nM; open triangles) or D-Ala²-Leu⁵-enkephalin (10 nM; open circles). The experiment has been replicated three times with similar results. With K_D values of 0.8 nM, the B_{max} values are 4.1, 2.4, and 2.5 fmol/mg of tissue for control, morphine, and D-Ala²-Leu⁵-enkephalin in curves, respectively. *B*, Rat brain homogenates were prepared, and binding was performed with $[^3\text{H}]\text{-}\beta\text{-endorphin}$ (0.02 to 1.7 nM) in the absence (solid circles) and presence (open circles) of both morphine (5 nM) and D-Ala²-Leu⁵-enkephalin (5 nM). The experiment has been replicated three times with similar results. With K_D values of 0.8 nM, the B_{max} are 4.7 and 2.2 fmol/mg of tissue in control and displaced studies, respectively.

TABLE IV

Inhibition of $[^3\text{H}]\text{-}\beta\text{-opioid}$ binding by naloxonazine

Rat brain membranes were incubated with naloxonazine (50 nM) or nothing at 25°C for 30 min and then washed three times before assaying with either $[^3\text{H}]\text{-}\beta\text{-endorphin}$ or $[^3\text{H}]\text{-dihydromorphine}$. Each wash consisted of an incubation at 25°C for 10 min followed by centrifugation ($49,000 \times g$ for 20 min) and resuspension. The results are the mean inhibition of binding from three separate experiments $\pm$ SEM.

	Inhibition of Binding by Naloxonazine
	%
$[^3\text{H}]\text{-}\beta\text{-Endorphin}$	38 ± 10
$[^3\text{H}]\text{-Dihydromorphine}$	54 ± 9

[^3H]- β -endorphin binding by morphine and D-Ala²-Leu⁵-enkephalin alone or together. In full saturation experiments, naloxonazine treatment inhibits approximately 55% of [^3H]- β -endorphin binding, yielding curves very similar to those seen using either morphine, enkephalin, or both agents as competitors (Fig. 5).

Discussion

The binding of [^3H]- β -endorphin to rat brain membranes is complex. The ability of unlabeled β -endorphin to displace the binding of a series of ^3H -labeled opioids fully (data not shown) implies that, at sufficiently high concentrations, β -endorphin will bind to virtually any type of opioid site. The important question is whether [^3H]- β -endorphin labels a β -endorphin-selective (ϵ) site or whether the binding merely reflects labeling of previously described sites, specifically μ and δ .

A number of other laboratories have reported that [^3H]- β -endorphin binds to brain and spinal cord membrane homogenates with K_D values ranging from 0.3 to 0.8 nM (Ferrara et al., 1979; Law et al., 1979; Akil et al., 1980, 1981; Ferrara and Li, 1980a, b; Hammonds and Li, 1981; Ho et al., 1983; Lin-Shiau et al., 1983), values very similar to the values found in our studies. Like the other groups (Ferrara et al., 1979; Law et al., 1979; Ferrara and Li, 1980a, b; Lin-Shiau et al., 1983), we also find that this binding is very sensitive to trypsin, *N*-ethylmaleimide, and sodium ions.

Examining the IC_{50} values of unlabeled β -endorphin, morphine, and D-Ala²-Leu⁵-enkephalin, we find that [^3H]- β -endorphin is displaced by itself more potently than by either of the other two compounds while being less effective against [^3H] dihydromorphine and [^3H]-D-Ala²-D-Leu⁵-enkephalin than morphine and D-Ala²-Leu⁵-enkephalin, respectively. These findings, which are very similar to those previously reported by the other groups, illustrate the differences between [^3H]- β -endorphin binding and the binding of either the μ ligand [^3H] dihydromorphine or the δ ligand [^3H]-D-Ala²-D-Leu⁵-enkephalin. These same IC_{50} values show that β -endorphin potently inhibits μ and δ binding. In addition, the competition studies of the opiates and opioid peptides against [^3H]- β -endorphin are multiphasic, strongly suggesting that [^3H]- β -endorphin is la-

beling more than one class of binding site despite the linear Scatchard plot.

It has been suggested that [^3H]- β -endorphin might be labeling both μ and δ sites, explaining the differences between [^3H]- β -endorphin binding and the binding of either [^3H] dihydromorphine or [^3H]-D-Ala²-D-Leu⁵-enkephalin (Lee and Smith, 1980). In this hypothesis, either compound alone would potentially displace the binding from one class of site, leaving the other class. For example, morphine would compete the μ sites, leaving [^3H]- β -endorphin binding to δ sites for which morphine has poor affinity. A similar situation would exist with the enkephalins. We have attempted to examine this possibility directly, and our results suggest that this is not the case. Rather, a portion of [^3H]- β -endorphin binding is quite sensitive to both morphine and an enkephalin and may correspond to the μ_1 site, while up to 20% of [^3H]- β -endorphin binding may correspond to μ_2 or δ sites. The remainder of the binding has a binding profile distinct from μ or δ sites and may represent a selective β -endorphin (ϵ) site.

This possibility is supported by evidence from several approaches. The competition curves of [^3H]- β -endorphin by either morphine or D-Ala²-Leu⁵-enkephalin alone or in the presence of a fixed concentration of the other provide the most graphic demonstration. Alone, both morphine and the enkephalin lower [^3H]- β -endorphin binding approximately 50% at a concentration of 5 nM. In the presence of the second compound at 5 nM, neither drug at 5 nM significantly lowers binding. These results strongly suggest that the binding of [^3H]- β -endorphin sensitive to low morphine concentrations is also quite sensitive to the enkephalins. Furthermore, the binding remaining in the presence of 5 nM concentration of either compound does not have the affinity for either morphine or D-Ala²-Leu⁵-enkephalin expected for either μ or δ sites.

The saturation studies yield similar results. Both morphine and the enkephalin inhibit [^3H]- β -endorphin binding to the same degree. Most important, the inhibition observed with the two of them together is no greater than that seen with either alone. Again, these findings imply that both morphine and the enkephalin are competing the same component of [^3H]- β -endorphin binding. The shape of these saturation curves in the presence of morphine and D-Ala²-Leu⁵-enkephalin might suggest that these compounds are not competitive inhibitors of [^3H]- β -endorphin binding. However, the K_D values of these drugs for this site based upon the full competition curves (Fig. 2) are well below 1 nM. Thus, the concentrations of the two compounds are 10-fold greater than their K_D values. This large excess of competitor would dramatically lower the observed affinity of [^3H]- β -endorphin for this site, and little binding to this component would be observed at the concentrations of [^3H]- β -endorphin used in the assays. To measure this binding, higher concentrations of [^3H]- β -endorphin would be needed. Thus, the reported curves cannot distinguish between competitive and the other types of inhibition.

In vivo studies have implicated a role of μ_1 sites in the analgesic and cataleptic actions of β -endorphin (Pasternak, 1981; Ling and Pasternak, 1982). The presence of a [^3H]- β -endorphin-binding component which is sensitive to both morphine and enkephalin raises the possibility that this portion of [^3H]- β -endorphin might be to μ_1 sites. Naloxonazine has been widely used as a μ_1 selective antagonist. In our binding studies, naloxonazine inhibits [^3H]- β -endorphin binding to the same extent as morphine and the enkephalin.

In conclusion, we feel that the [^3H]- β -endorphin binding observed under these conditions primarily consists of two major components. One appears to correspond to the μ_1 site, while the other does not possess characteristics suggestive of either μ or δ sites. This second site with its higher affinity for β -endorphin over a number of other opioids might represent a

Figure 5. Saturation of [^3H]- β -endorphin binding in control and naloxonazine-treated tissue. Rat brain homogenates were prepared and were then treated with naloxonazine (50 nM) and washed as previously described (Hahn et al., 1982). Binding with [^3H]- β -endorphin (0.4 to 2.3 nM) in control (solid circles) and naloxonazine-treated (open circles) tissue was then determined. Points represent the means $\pm$ SEM of triplicate samples from a representative experiment. The experiment has been replicated three times with similar results. With K_D values of 0.8 nM, the B_{max} values are 5.7 and 2.4 fmol/mg of tissue for control and naloxonazine-treated tissue, respectively.

selective β -endorphin, or ϵ , site within the central nervous system. Autoradiographical studies (Goodman et al., 1983) also suggest different regional distributions of [³H]- β -endorphin, [³H]dihydromorphine, and [³H]-D-Ala²-D-Leu⁵-enkephalin binding in rat brain, consistent with the proposal of specific β -endorphin-selective binding sites within the central nervous system. Other groups have also noted that the binding of [³H]- β -endorphin does not correspond to either μ or δ binding (Ferrara et al., 1979; Law et al., 1979; Akil et al., 1980; Ferrara and Li, 1980a, b; Hammonds et al., 1981; Ho et al., 1983; Lin-Shiau et al., 1983). However, they have not noted the presence of the binding component sensitive to both morphine and D-Ala²-Leu⁵-enkephalin which may correspond to μ_1 sites. Clearly, [³H]- β -endorphin will label some μ_2 and δ sites at the concentrations employed in our assays, but this binding appears to be a small part of total [³H]- β -endorphin binding.

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