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BINDING OF ^{3}H-β-ENDORPHIN TO RAT BRAIN MEMBRANES: CHARACTERIZATION OF OPIATE PROPERTIES AND INTERACTION WITH ACTH

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The binding of tritiated β-endorphin (^{3}H-β-EP) to brain homogenates is described. This had been difficult to achieve due to the lack of availability of ^{3}H-β-EP and to technical difficulties associated with high non-specific binding of β-EP. We now report that ^{3}H-β-EP binding is saturable, stereospecific, has high affinity and is inhibited by sodium. Its dissociation rate is ten-fold longer than that of naloxone. Its regional distribution exhibits interesting differences from naloxone and enkephalin binding. $ACTH_{1-24}$ appears to displace it more effectively than it displaces ^{3}H-naloxone. The results are discussed in terms of multiple transmitter systems and the multiple opiate receptor hypothesis.

^{3}H-β-Endorphin binding Opiate receptors Peptide binding

1. Introduction

While the endogenous opioid, β-endorphin (β-EP) was first discovered in the pituitary (Li and Chung, 1976; Bradley et al., 1976), it is also present within an anatomically distinct neuronal system in mammalian brain. We and others (Watson et al., 1978; Bloom et al., 1978) have shown to existence of β-EP and its precursors in a well-defined hypothalamic cell group which projects to a number of limbic and midbrain structures. This system is independent of the pituitary β-EP system, and is readily distinguished from enkephalin pathways (Watson et al., 1978). Interestingly, the synthetic machinery characteristic of pituitary β-EP — including the 31K prescursor described by Mains et al. (1977) — appears to exist within the brain. This conclusion is based primarily on immunohistochemical evidence demonstrating β-LPH, ACTH and a number of other 31K fragments (Watson et al., 1978) within these β-EP-containing neurons.

β-EP is known to exhibit opiate-like properties both in vitro (Cox et al., 1976) and in vivo (Loh et al., 1976). However, in pharmacological doses, it also produces effects which differ, in part, from those of morphine. These include the induction of limbic seizures and rigidity at subanalgesic doses (Bloom et al., 1976). While ^{3}H-enkephalin binding had been studied and characterized, we know little about the specific properties of β-EP binding. ^{3}H-β-EP has not been commercially available to date, preventing direct studies of its receptor interactions. Yet, the unique anatomical and biosynthetic properties of the brain β-EP system, and the distinct pharmacological pro-

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perties of β-EP warrant a closer examination of its interaction with a brain receptor. Whether such a receptor would prove to be identical or in some way discernable from the classical 'opiate receptor' (Martin et al., 1976) would shed light on the function of β-EP and on the hypothesis of multiple opiate receptors. In this paper, we describe ^{3}H-β-EP binding to rat brain membranes, including a characterization of 'opiate properties', a mapping of regional distribution and interaction with ACTH — another 31K product.

2. Materials and methods

Tritium-labelled β_h-EP was synthesized according to a previously described procedure (Houghten and Li, 1978), achieving a specific activity of 50 Ci/mmole. The binding assay is descibed in greater detail elsewhere (Hewlett, Akil, Barchas and Li, in preparation). Briefly, whole rat brains minus cerebella are homogenized in 12 vol of Tris HCl (pH 7.8 at 25°C) and centrifuged at 35000 × *g* for 30 min. The pellet is then resuspended in buffer and allowed to incubate for 1 h at room temperature. It is then recentrifuged and suspended in the same volume. ^{3}H-β-EP (0.025 ml) at varying concentrations is incubated with 0.2 ml of this membrane preparation in the presence or absence of unlabelled β-EP (in 0.025 ml). To determine saturable and non-specific binding, a 2 μM concentration of unlabelled β-EP is routinely used. Incubation is carried out for 2—3 hours at 0°C. Because of β-EP's great propensity to adhere to surfaces, separation of bound from free ^{3}H-β-EP is extremely critical and technically difficult. Good separation is achieved in one of two ways: the first involves centrifugation in a Sorvall centrifuge swing bucket rotor (HS_4) at 7000 rpm for 20 min. The supernatant is then aspirated, the pellet resuspended in standard buffer and transferred to a vial for liquid scintillation counting. The second separation procedure employs rapid filtration and washing on precoated GF/B Whatman filters, which are then solubilized and counted. The coating of filters with BSA/polylysine (5 ml of 0.01% polylysine/0.4% BSA) is critical for detecting saturable binding. Without coating, over 50% of ^{3}H-β-EP binds non-specifically to the filters. Both techniques yield similar results, although the ratio of displaceable saturable counts to the total counts is better with filtration (about 60—80% at 3 nM) than it is with centrifugation (30—50% at 3 nM). In both techniques, the standard error of the replicates is approximately 1%.

3. Results

3.1. Characterization

Fig. 1 shows a Scatchard plot of ^{3}H-β-EP binding in whole brain. β-EP was added in concentrations ranging between 0.5 nM and

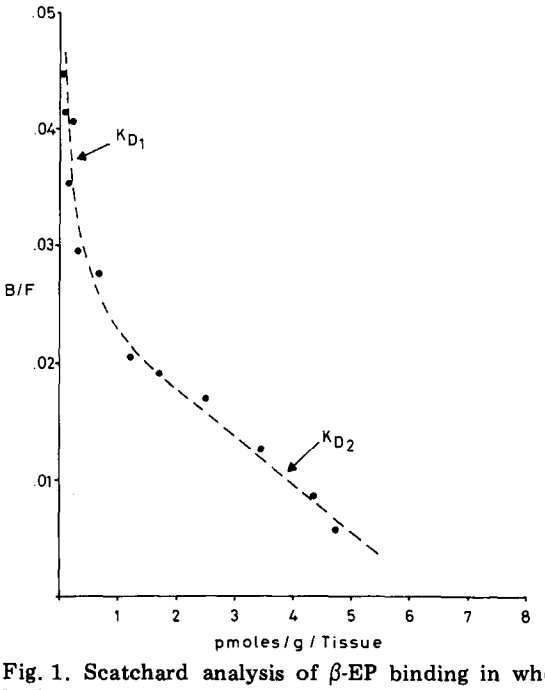

Fig. 1. Scatchard analysis of β-EP binding in whole brain at .05, .16, .25, 0.75, 1.6, 4, 6, 10, 18, and 38 nM concentrations. Assay carried out at 0°C for 2.5 h — separation by filtration. K_{D1} = 1.02 nM, K_{D2} = 16.73 nM.

TABLE 1

Time course of β-EP binding at 0°C.

Time (min)	Specific DPM's bound	Specific/ total X 100
1	853 ± 40	37%
5	1095 ± 65	52%
10	2030 ± 100	57%
30	3784 ± 63	78%
60	4240 ± 150	75%
120	5084 ± 202	66%
180	4681 ± 175	62%
240	4841 ± 160	64%
24 h	3966 ± 100	70%

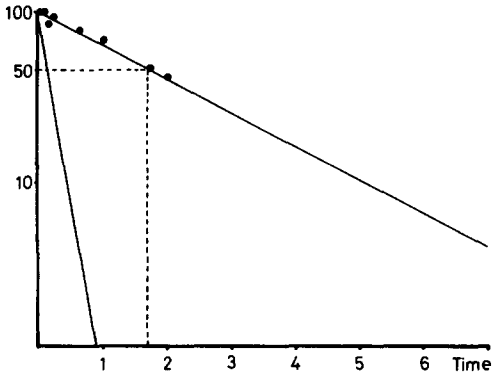

Fig. 2. Dissociation rate of ^{3}H-naloxone and ^{3}H-β-EP under identical conditions. After equilibrium was achieved, dissociation was brought about by adding a thousand-fold concentration of unlabeled ligand and filtering at various time points (h) thereafter (*abscissa*). The curve with filled circles represent the ^{3}H-β-EP results. The unmarked line represents ^{3}H-naloxone dissociation (an equal number of points was studied with ^{3}H-naloxone but those can only be visualized with a different scale, due to the big difference in time course). Incubation at 0°C; β-EP t½ = 90 min; naloxone t½ = 8.5 min. Ordinate: % control.

65 nM. The tissue was incubated at 0°C for 150 min, and bound β-EP was separated by filtration. Scatchard analysis demonstrates a curvilinear plot which can be resolved into two components, with two distinct affinities and capacities ($KD_1 = 1.0 \times 10^{-9}$ M; $KD_2 = 16.7 \times 10^{-9}$ M). Similar results are obtained by centrifugation. Displacement curves with unlabelled β-EP and binding with varying concentrations of labelled material yield similar estimates of affinity and capacity of binding. The total binding capacity in whole brain is estimated at 6.8 pmoles/g of tissue in the illustrated experiment (and has ranged between 6.8 and 13.7 pmoles/g in other experiments) — significantly lower than what is routinely obtained with ^{3}H-naloxone binding in the same preparation. Whether this discrepancy is entirely due to breakdown or to a true difference in receptor number remains to be determined.

In three separate studies, we examined the time course of ^{3}H-β-EP binding (table 1). The labelled peptide was incubated in the presence or absence of 2 μM unlabelled β-EP for various time periods ranging from 1 min to 24 h. The results indicate that at 0°C equilibrium is reached at 2 h and remains relatively constant for 24 h. The slight decline seen in some preparations at later time points is probably due to some enzymatic breakdown of ^{3}H-β-EP by the membrane preparation. The best ratio of specific/total DPM is, in fact, achieved at 30 minutes, rather than at equilibrium.

The rate of dissociation of ^{3}H-β-EP from the binding site was estimated by displacing the labelled compound with a high concentration of unlabelled β-EP (fig. 2). Samples containing 2 nM ^{3}H-β-EP were allowed to incubate to equilibrium for 2 h, at which point estimates of total and non-specific binding were obtained. Unlabelled β-EP at a thousand-fold higher concentration (2 μM) were then added. The samples were filtered at several time points thereafter (1 min to 3 h), with appropriate controls. A plot of log percent specific binding versus time yielded an estimate of t1/2 = 90.33 ± 5 min, which is significantly longer than the t1/2 for naloxone under identical conditions (t1/2 = 9 min) (fig. 2).

At higher temperatures, ^{3}H-β-EP binding is decreased rather than enhanced, also pointing

to enzymatic breakdown of the opioid. While equilibrium at 25°C is achieved more rapidly, the number of specific DPM's bound at 25°C is only 30% of specific binding at 0°C when the incubation is allowed to proceed for 2 h. No specific binding could be seen at 37°C with 2 h incubations.

The effect of tissue concentration on specific binding was examined in two separate studies. Brain homogenates were resuspended in 6, 12, 24 or 36 vol of buffer and tested in the binding assay. The results showed a linear relation between tissue concentration and specific binding, such that binding doubled as the tissue concentration was doubled.

The opiate nature of this binding was investigated in a number of studies. The ability of naloxone, levorphanol, dextrorphan, leucine enkephalin (Leu-E) and methionine enkephalin to displace 3 nM of ^{3}H-β-EP was studied both in whole brain and in brain parts. In all cases, 1 μM of the opiate agonists or antagonists produced between 90 and 100% displacement of saturable β-EP binding. Dextrorphan, on the other hand, used at the same concentration, left this binding unaltered. These results suggest that the majority of ^{3}H-β-EP is binding to the stereospecific opiate receptor.

The relative potencies of β-EP and Leu-E are quite different depending on whether they are competing against ^{3}H-Leu-E or ^{3}H-β-EP. While β-EP is 13-fold more potent than Leu-E in displacing ^{3}H-β-EP (β-EP $IC_{50} = 3.5 \times 10^{-9}$ M; Leu-E $IC_{50} = 45 \times 10^{-9}$ M), it is half as potent as Leu-E in displacing ^{3}H-Leu E binding (β-EP $IC_{50} = 20.5 \times 10^{-9}$ M; Leu E $IC = 9 \times 10^{-9}$ M).

Another characteristic of opiate binding is the 'sodium effect'. As an opiate agonist, β-EP binding would be expected to decrease in the presence of sodium (Pert and Snyder, 1974; Simon et al., 1973, 1975). In fact, 100 mM NaCl produces a total inhibition of all specific binding of 4 nM ^{3}H-β-EP. Ten mM NaCl resulted in 57% inhibition of binding.

In order to study the regional distribution of ^{3}H-β-EP binding, 18 rats were sacrified and their brains dissected into the following regions: hypothalamus, striatum, hippocampus/septal region, cortex, medulla-pons, midbrain, thalamus, cerebellum and pituitary (Holman et al., 1976, see table 2). ^{3}H-β-Ep binding was studied at two concentrations, 3.25 nM and 33 nM, in order to tap both the high and low affinity components of the binding.

Both concentrations yielded a roughly similar profile across regions, with striatum exhib-

TABLE 2

Specific ^{3}H-β-EP and ^{3}H-naloxone binding across rat brain regions, in the same preparations (pmoles/g tissue).

	^{3}H-β-EP		^{3}H-Naloxone	
	3.25 nM	33.25 nM	3.25 nM	33.25 nM
Striatum	2.82	19.82	10.33	60.62
Cortex	1.50	8.50	8.03	37.04
Hypothalamus	1.58	7.08	8.78	53.27
Hippocampus/ septum	2.44	14.96	6.90	36.00
Midbrain/ thalamus	1.30	4.22	9.77	61.93
Medulla/pons	0.76	5.78	4.34	29.30
Cerebellum	—	—	—	—
Pituitary	—	—	—	—

TABLE 3

Effect of $ACTH_{1-24}$ (10 μM) on ^{3}H-opiate ligands (3 nM).

	^{3}H-β-EP	^{3}H-Naloxone	^{3}H-Met-enkephalin
Expt. 1			
Control DPM	5285	4846	4997
ACTH added	2383	3191	3020
% Control	45%	66%	61%
Expt. 2			
Control	3754	3599	—
ACTH added	1881	2671	—
% Control	50%	74%	—

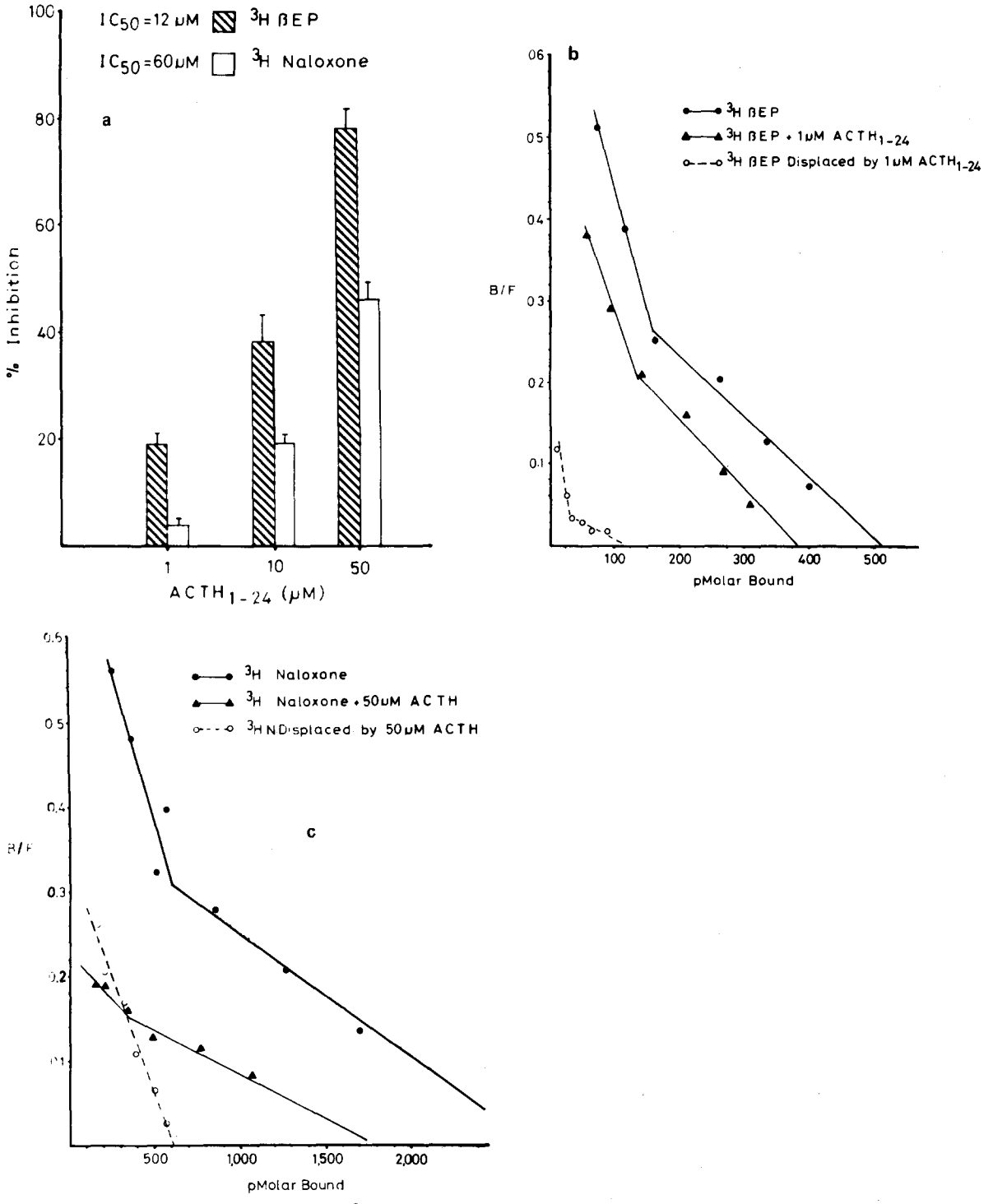

Fig. 3. Interaction of $ACTH_{1-24}$ with ^3H-opiate ligands. Panel 3a represents the dose-dependent inhibition of opiate binding by $ACTH_{1-24}$. As can be seen, ^3H-β-EP binding is significantly more inhibited by $ACTH_{1-24}$ than is ^3H-naloxone binding ($P < 0.01$). 3b. Scatchard plot analysis of ^3H-β-EP in the presence or absence of 1 μM $ACTH_{1-24}$, showing inhibition of both high and low affinity components of ^3H-β-EP by ACTH. The displaced counts at each concentration of ^3H-β-EP (i.e. the difference between the two curves) is indicated by the dashed lines. Similar results are seen with 10 and 50 μM $ACTH_{1-24}$. 3c. Parallel to 3b, showing ACTH displacement of ^3H-naloxone. Note that 50 μM ACTH is depicted here, although similar results are obtained with 1 and 10 μM ACTH. The graph shows little change in the low affinity component of naloxone, and the difference curve (dashed lines) depicts that only high affinity naloxone binding is displaced by $ACTH_{1-24}$.

iting the highest binding and cerebellum exhibiting little or no specific binding. The rank order of the regions is roughly similar to what we commonly see with binding of other opiates (cf. table 2, Pert and Snyder, 1973). However, there are also interesting differences — such as the relatively high binding of β-EP in hippocampus — a result not obtained with either ^{3}H-naloxone or ^{3}H-enkephalin. While the unique profile may be due to differential breakdown, our experiments suggest that under our conditions, breakdown of ^{3}H-β-EP is minimized.

3.2. ACTH interactions

Since β-EP is synthesized from a common precursor molecule containing the structure of $ACTH_{1-39}$, α-MSH and a number of related peptides, and since Terenius et al. (1975) had demonstrated some interaction of ACTH-related peptides with the opiate receptor, we tested for possible interactions between 31K-related substances and ^{3}H-β-EP. α-MSH, β-MSH, $ACTH_{4-9}$, and modified $ACTH_{4-9}$ (Org 2766), and DTγE were ineffective at inhibiting ^{3}H-β-EP binding at concentrations of up to 10 μM. On the other hand, $ACTH_{1-24}$ exhibited dose dependent inhibition of ^{3}H-β-EP. The IC_{50} for $ACTH_{1-24}$ is 12 μM, obviously significantly higher than IC_{50}'s of opiate peptides or alkaloids.

Nonetheless, the interaction appeared unique to $ACTH_{1-24}$. It was therefore of interest to determine whether ACTH would displace all opiate binding equally, or whether it would display closer interaction with β-EP. Table 3 shows that $ACTH_{1-24}$ appears to displace ^{3}H-β-EP somewhat more effectively than it did ^{3}H-naloxone or ^{3}H-Met-enkephalin. Therefore, we undertook a more thorough comparison of $ACTH_{1-24}$ effect on ^{3}H-β-EP and ^{3}H-naloxone binding. Full concentration curves for ^{3}H-β-EP and ^{3}H-naloxone were carried out under four conditions: in the absence of any $ACTH_{1-24}$, or in the presence of 1 μM, 10 μM and 50 μM $ACTH_{1-24}$. Inhibition by each concentration of $ACTH_{1-24}$ at each concentration of labelled ligands was computed, and the data were transformed for Scatchard plot analysis. Fig. 3a demonstrates that throughout the range of labelled concentrations $ACTH_{1-24}$ produced a dose dependent inhibition of both ^{3}H-naloxone and ^{3}H-β-EP. However, it consistently produced higher inhibition against ^{3}H-β-EP. Scatchard analyses of the results showed that all concentration of $ACTH_{1-24}$ inhibited both high and low affinity components of ^{3}H-β-EP binding (fig. 3b). However, $ACTH_{1-24}$, even at concentrations up to 50 μM inhibited primarily the high affinity but not the low affinity component of ^{3}H-naloxone binding (fig. 3c).

4. Discussion

Thus, ^{3}H-β-EP exhibits high affinity saturable binding to rat brain membranes. This binding bears striking similarities to the 'opiate receptor' in that it is displaced stereospecifically by opiate alkaloids and is sensitive to sodium. Interestingly, Hazum et al. (1979) have recently characterized the binding of iodinated [D-Ala^2]β-EP to brain membranes. They have noted that the binding of this peptide is not inhibited by 100 mM sodium chloride. The contrast between the two findings appears to be due to the D-Ala substitution, since in our hand both ^{3}H-β-EP and ^{125}I-β-EP are highly sensitive to sodium (Akil and Lin, in preparation). This suggests that the substitution of glycine by D-Alanine brings about changes in the way the β-EP analogue interacts with the opiate receptor.

The present results suggest that the two classes of endogenous opioid ligands identified to date — the enkephalins and β-EP — are recognized by membrane macromolecules which share a common structural unit, leading to a great deal of 'cross reactivity'. It is possible that the specificity of the interactions between opiate receptors and endogenous opiates resides primarily in the anatomical circuitry, or in post-synaptic events. Nonetheless, the notion of 'multiple opiate receptors' is

given partial support by the findings that β-EP and enkephalin exhibit very different relative potencies depending on whether they are competing against ^{3}H-enkephalin or ^{3}H-β-EP. The differential regional distribution in the binding of ^{3}H-β-EP and ^{3}H-naloxone also points to a partial separation of subclasses of opiate receptors.

The finding that $ACTH_{1-24}$ interacts at micromolar concentration with opiate receptors is consistent with the previous report of Terenius et al. (1975). The present is an extension showing that $ACTH_{1-24}$ discriminates between ^{3}H-β-EP and ^{3}H-naloxone, inhibiting both high and low affinity binding components of the former and only the high affinity component of the latter. This is consistent with the notion that ACTH and β-EP are more closely related, due to their common biosynthetic origin. However, the physiological significance of this interaction is difficult to ascertain, since high concentrations are required for the competition.

β-EP appears to dissociate extremely slowly from its binding site, a finding which is congruent with its high potency and long time course upon in vivo administration. Such slow dissociation has important implications for considering its mode of action in the brain. Since classical neurotransmitters are thought to produce rapid and short-lived effects on the postsynaptic neurons, a longer-acting endogenous ligand could be construed as serving significantly different neuromodulatory functions.

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