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Naloxone-inaccessible σ receptor in rat central nervous system

(multiple opiate receptors/ethylketocyclazocine/phencyclidine/SKF 10,047/psychosis)

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ABSTRACT **It has been postulated that the psychotomimetic effects of opiates of the benzomorphan series are due to their activity at the σ receptor. Therefore, the binding of (±)-[^{3}H]ethylketocyclazocine ([^{3}H]EKC), a benzomorphan, to synaptosomal membranes of rat central nervous tissue was studied. Surprisingly, high concentrations of naloxone, a μ, δ, and κ receptor antagonist, only inhibited about 80% of the specifically bound [^{3}H]-EKC in the spinal cord. This suggested that the remaining 20% of the binding sites were not μ, δ, or κ. The Scatchard plot of the binding of [^{3}H]EKC was nonlinear but became linear in the presence of naloxone (1 μM), suggesting a single class of naloxone-inaccessible receptor sites. This biochemically readily distinguishable receptor type bound the dextrorotatory isomer of EKC stereoselectively. The σ agonist *N*-allylnormetazocine [(+)-SKF 10,047] stereoselectively competed with the binding of [^{3}H]EKC to this naloxone-inaccessible binding site. A number of opiates that have psychotomimetic activity also competed for binding to this binding site. This binding site is designated as σ binding site according to the nomenclature originally suggested by Martin *et al.* [Martin, W. R., Eades, C. G., Thompson, J. A., Huppler, R. E. & Gilbert, P. E. (1976) *J. Pharmacol. Exp. Ther.* 197, 517–532]. The drug selectivity and regional distribution of this σ binding site in the rat central nervous system are different from that of the μ and δ opioid receptors and phencyclidine receptors. The concentration of the σ binding site is highest in the spinal cord, pons and medulla, and cerebellum.**

Opiates have a wide range of pharmacological effects. To account for the different pharmacological effects of some opiates, multiple opioid receptors were hypothesized (1, 2). The four opiate receptor types that have been proposed are μ, δ, κ, and σ. It has been proposed that μ receptors mediate analgesia; κ receptors mediate analgesia and sedation; δ receptors mediate satisfaction, reward, and seizure; and σ receptors mediate psychotomimetic effects. Of these, the μ and δ receptors have been well characterized biochemically (2–7). Recently, increasing biochemical evidence also suggests the existence of a κ receptor (7). However, there is little biochemical evidence of a σ receptor. This is in part due to the lack of a σ-specific ligand, because the prototype σ agonist SKF 10,047 also binds the other opiate receptor types. The present paper describes the identification and characterization of a biochemically distinguishable σ binding site in the rat central nervous system (CNS). This binding site is not blocked by naloxone.

MATERIALS AND METHODS

(±)-[^{3}H]Ethylketocyclazocine ([^{3}H]EKC) (21.8 Ci/mmol; 1 Ci = 3.7×10^{10} Bq), [^{3}H]naloxone (40.0 Ci/mmol), [^{3}H][D-Ala^2,D-Leu^5]enkephalin (39.5 Ci/mmol), (+)-[^{3}H]SKF 10,047 (34.0 Ci/mmol), and [^{3}H]phencyclidine ([^{3}H]PCP) (48.0 Ci/mmol) were from New England Nuclear. Male Sprague–Dawley rats (Charles River Breeding Laboratories) at 200–250 g were sacrificed by decapitation. Rat brain regions were dissected according to Heffner *et al.* (8). Brain and spinal cord membranes were prepared by a modified procedure of that described by Blume (9). The brain and spinal cord tissues were homogenized (20 s) in 10 vol (wt/vol) of ice-cold 0.32 M sucrose with a Brinkman Polytron (setting 8). The homogenate was centrifuged at $920 \times g$ for 10 min at 4°C. The supernatant was centrifuged at $47,000 \times g$ for 20 min. The resulting membrane pellet was resuspended in 10 vol (original wt/vol) of 50 mM Tris·HCl (pH 7.4) and incubated at 37°C for 30 min to degrade and dissociate bound endogenous ligands. The membranes were then centrifuged at $47,000 \times g$ for 20 min and resuspended in 50 mM Tris·HCl. Membranes (1.5–2.0 mg of protein) were incubated with radiolabeled ligands in 50 mM Tris·HCl (pH 7.4) containing 100 mM NaCl at a final volume of 2 ml for 45 min at room temperature, rapidly filtered through Whatman GF/C glass filters, and washed three times with ice-cold Tris·HCl (5 ml). In the [^{3}H][D-Ala^2,D-Leu^5]enkephalin binding experiments, NaCl was not present. Nonspecific binding of [^{3}H]naloxone and [^{3}H][D-Ala^2,D-Leu^5]enkephalin was determined in the presence of 10 μM naloxone. Nonspecific binding of [^{3}H]EKC and (+)-[^{3}H]SKF 10,047 was determined in the presence of 10 μM EKC. The same (+)-[^{3}H]SKF 10,047 nonspecific binding was obtained by using 10 μM SKF 10,047. Nonspecific binding of [^{3}H]PCP was determined in the presence of 100 μM PCP. Protein concentrations were determined by the Lowry method (10).

RESULTS

[^{3}H]EKC bound rat spinal cord membranes specifically. Morphine (μ agonist), [D-Ala^2,D-Leu^5]enkephalin (δ agonist), and their equimolar combinations at concentrations up to 0.1 mM did not completely inhibit the specifically bound [^{3}H]EKC (Fig. 1). Surprisingly, naloxone, a μ, δ, and κ antagonist, could only maximally inhibit 80% of the bound [^{3}H]EKC. These data suggested that the remaining 20% of the naloxone-inaccessible binding sites were not μ, δ, or κ opiate receptor types. In subsequent experiments, this naloxone-inaccessible site was studied by performing binding in the presence of 1 μM naloxone to eliminate binding of [^{3}H]EKC to μ, δ, and κ receptor types. Typical experiments with [^{3}H]EKC in the presence of excess unlabeled naloxone gave 900–1,200 dpm total binding and 450–600 dpm nonspecific binding. A second species was examined. It was found that guinea pig brain membranes had a higher concentration of σ binding sites. By using 1 nM (+)-[^{3}H]SKF 10,047 to label the σ binding sites and guinea pig brain membranes with less protein compared to the rat spinal cord membranes used, a typical experiment gave 800 dpm total binding and 140 dpm nonspecific binding. The properties of σ binding sites in

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Abbreviations: EKC, ethylketocyclazocine; CNS, central nervous system; PCP, phencyclidine.

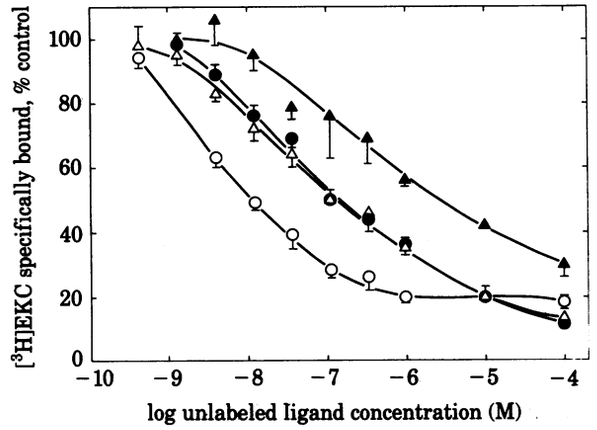

FIG. 1. Competitive inhibition of the binding of 5 nM (±)-[^{3}H]EKC to rat spinal cord membranes by naloxone (○), morphine (●), [D-Ala^2,D-Leu^5]enkephalin (▲), and equimolar combinations of morphine and [D-Ala^2,D-Leu^5]enkephalin (△). Binding was performed as described in the text in the presence of 100 mM NaCl. Data represent the average and range of two experiments in duplicate. Three other experiments using 6 nM [^{3}H]EKC were performed with similar results.

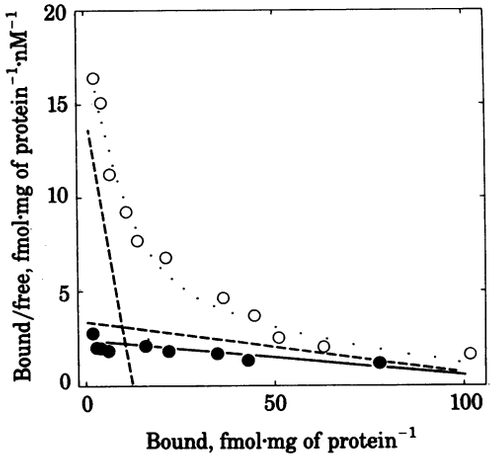

FIG. 3. Scatchard plot of the binding of (±)-[^{3}H]EKC to rat spinal cord membranes in the absence (○) and presence of 1 μM naloxone (●). Binding was performed as described in the text in the presence of 100 mM NaCl. Data represent the mean of three experiments in duplicate. ---, Computer-resolved linear components of the experimental data obtained in the absence of naloxone; ·····, summation of the two linear components; ——, obtained by linear regression of the (●) data points.

the guinea pig brain membranes will be published elsewhere. Binding of [^{3}H]EKC to this naloxone-inaccessible site reached a maximum at 15 min (Fig. 2).

The Scatchard plot of the binding of [^{3}H]EKC to rat spinal cord membranes was nonlinear (Fig. 3), suggesting at least a class of high-affinity sites with K_d = 0.86 nM and B_{max} = 12 fmol/mg of protein and a class of low-affinity sites with K_d = 36 nM and B_{max} = 126 fmol/mg of protein. In the presence of 1 μM naloxone, the Scatchard plot became linear, showing only the low-affinity sites with similar dissociation constant and number of sites (K_d = 54 nM and B_{max} = 133 fmol/mg of protein). These data suggested that the [^{3}H]EKC naloxone-inaccessible binding site was a low-affinity site.

The effect of drugs on competition with the binding of [^{3}H]-EKC to the naloxone-inaccessible site is shown in Fig. 4. (+)-EKC was very effective in competing with [^{3}H]EKC for binding to this site, whereas (−)-EKC was at most 1/200th as potent. These data suggested that it was the (+)-isomer of [^{3}H]-EKC that bound the naloxone-inaccessible site. (+)-SKF 10,047, a σ receptor agonist, was also very effective in competing for binding to this site.

A comparison of the relative potencies of drugs in inhibiting (+)-[^{3}H]SKF 10,047 and [^{3}H]PCP binding and the binding of [^{3}H]EKC to the naloxone-inaccessible sites is shown in Table 1. A number of opiates that produce apparent psychotomimetic effects in man were potent in inhibiting the binding of [^{3}H]EKC to this binding site. Opiates that have low or no apparent psychotomimetic effect were not active towards this binding site. Haloperidol had the highest affinity for this binding site. However, this [^{3}H]EKC naloxone-inaccessible binding site was not a dopamine receptor because (+)-butaclamol, a stereoselective dopamine receptor antagonist, was 1/28th as potent as (−)-butaclamol. Other dopaminergic compounds such as clozapine and sulpiride were essentially inactive. The β-adrenergic antagonist, propranolol and two histamine H_1 receptor antagonists had

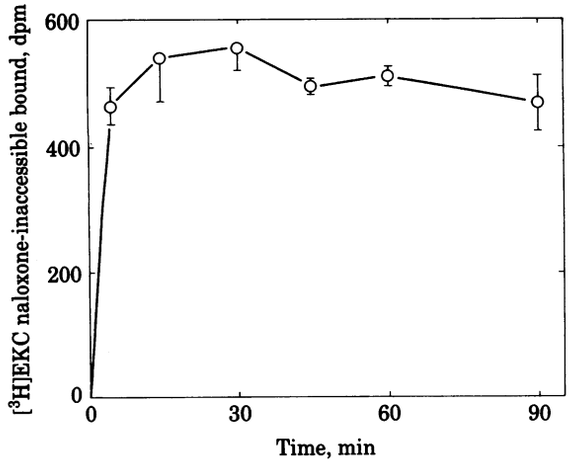

FIG. 2. Time course of (±)-[^{3}H]EKC naloxone-inaccessible specific binding to rat spinal cord membranes. Binding was performed as described in the text in the presence of 100 mM NaCl, 1 μM naloxone, and 5 nM [^{3}H]EKC. Data represent the mean ± SEM of three experiments in duplicate.

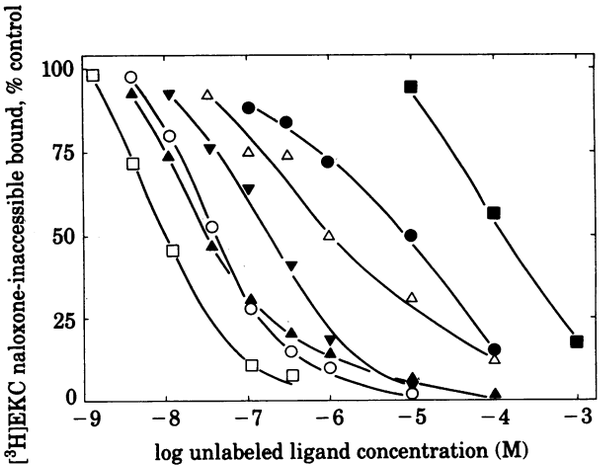

FIG. 4. Competitive inhibition of 5 nM (±)-[^{3}H]EKC naloxone-inaccessible specific binding in rat spinal cord membranes by haloperidol (□), (+)-SKF 10,047 (▲), (−)-SKF 10,047 (△), (+)-EKC (○), (−)-EKC (●), (±)-bremazocine (▼), and (−)-morphine (■). Binding was performed as described in the text in the presence of 100 mM NaCl and 1 μM naloxone. Data represent the average of three to seven experiments in duplicate.

Table 1. Relative potencies of drugs in inhibiting (±)-[^{3}H]EKC naloxone-inaccessible, (+)-[^{3}H]SKF 10,047, and [^{3}H]PCP specific binding to rat spinal cord membranes

Compound	IC_{50}, nM		
	[^{3}H]EKC and naloxone*	(+)-[^{3}H]SKF†	[^{3}H]PCP‡
Opiates			
(±)-Pentazocine	23 ± 3 (7)		
(+)-SKF 10,047	32 ± 8 (5)	50 ± 8 (4)	7,500 ± 3,300 (3)
(+)-EKC	42 ± 5 (5)	64 ± 13 (4)	63,000 ± 21,000 (3)
(±)-Cyclazocine	67 ± 12 (5)		
(±)-Bremazocine	190 ± 32 (4)		
(±)-U50,488H	520 ± 100 (5)		
(−)-SKF 10,047	1,000 ± 80 (5)	1,200 ± 540 (4)	13,000 ± 6,800 (3)
(−)-EKC	14,000 ± 5,100 (6)	22,000 ± 6,800 (2)	66,000 ± 19,000 (3)
(−)-Nalorphine	13,000 ± 1,400 (4)		
Naloxone	>100,000 (3)	>100,000 (2)	>10,000 (2)
Others			
Haloperidol	12 ± 4 (5)	6 ± 2 (4)	5,000 ± 1,500 (3)
(−)-Butaclamol	50 ± 10 (3)	39 ± 8 (4)	>10,000 (2)
Pyrilamine	110 ± 20 (4)	142 ± 38 (4)	
Promethazine	190 ± 27 (2)		
Thioridazine	235 ± 40 (4)		
Propranolol	320 ± 80 (4)		
Chlorpheniramine	560 ± 120 (3)		
Mianserin	630 ± 20 (5)		
(+)-Butaclamol	1,400 ± 250 (3)		
PCP	2,400 ± 1,200 (3)	1,470 ± 490 (4)	785 ± 170 (4)
Clozapine	18,000 ± 2,400 (4)		
Atropine	>10,000 (2)		

Drugs that did not bind the [^{3}H]EKC naloxone-inaccessible sites (IC_{50} > 0.1 mM) were (−)-morphine, (−)-etorphine, (−)-diprenorphine, [D-Ala^2,D-Leu^5]enkephalin, dynorphin-(1–13), pilocarpine, and sulpiride. Other inactive drugs with IC_{50} > 1 mM were hexamethonium, tubocurcarine, diazepam, serotonin, and methylsergide. Binding was performed in the presence of 100 mM NaCl. Values are means ± SEM with the number of experiments done in duplicate given in parenthesis. IC_{50}s were determined from log-logit plots.

*5 nM (±)-[^{3}H]EKC in the presence of 1 μM naloxone.

†2 nM (+)-[^{3}H]SKF 10,047.

‡5 nM [^{3}H]PCP.

moderate activity, whereas ligands for other major receptor types were essentially inactive. Thus, it appears that this [^{3}H]EKC naloxone-inaccessible receptor binds opiates that produce psychotomimetic effects. The drug specificity of the (+)-[^{3}H]SKF 10,047 binding sites appeared to be identical to that of the [^{3}H]EKC naloxone-inaccessible binding sites but different than that of the [^{3}H]PCP binding sites (Table 1). Thus, it appears that (+)-[^{3}H]SKF 10,047 and [^{3}H]EKC in the presence of excess naloxone were labeling the same sites. (+)-[^{3}H]SKF 10,047 and (+)-[^{3}H]EKC were found to be stereoselective ligands for the same σ binding sites in the guinea pig brain homogenates (unpublished data).

The regional distribution of the σ binding sites in the rat CNS was determined by using (+)-[^{3}H]SKF 10,047 as the radioactive ligand. The regional distribution of this σ binding site was different from the regional distribution of the μ and δ opioid receptors and the PCP receptors (Table 2). The regional distribution of [^{3}H]PCP did not change when binding was performed in the absence of NaCl but the amount bound was about twice as much (data not shown). The concentration of this σ binding site in the rat was highest in the spinal cord, pons and medulla, and cerebellum. Another major difference was the relatively high concentration of σ binding sites in the anterior pituitary, where there were essentially no μ or δ receptors.

Drug specificity of the σ binding sites from rat spinal cords and brains was compared to determine if the sites were similar in the two tissues. Preliminary results suggest that the binding sites from both tissues had similar drug specificity (Table 3).

DISCUSSION

The data suggest the existence in the rat CNS of a type of receptor site that is biochemically and topographically distinct from the κ opioid receptor and the well-characterized μ and δ opioid receptors. This σ receptor binds (+)-EKC and (+)-SKF 10,047 stereoselectively. Other opiates such as pentazocine, cyclazocine, and bremazocine, which bind the μ and δ receptors, also bind the σ receptor with moderate affinities. These benzomorphans produce a combination of sedation, "drunkenness," or psychosis differing from any morphine effect (11, 12). The psychotomimetic effects include depersonalization, dysphoria, suspiciousness, and hallucinations (13). U50,488H, a putative κ agonist, also has moderate affinity for this binding site. The typical μ agonist morphine, the typical δ agonist [D-Ala^2,D-Leu^5]enkephalin, and the endogenous κ agonist dynorphin-(1–13) essentially do not bind this σ binding site.

Haloperidol, which has high affinity for the σ binding site, was very effective in blocking the discriminative properties of PCP in the rat (14). If the PCP receptor and the σ receptor are the same receptor, then haloperidol may be an antagonist for the σ receptor. However, it has also been reported that neither naloxone nor haloperidol could antagonize the discriminative stimulus produced by SKF 10,047 in the rat, though PCP readily generalized to SKF 10,047 in that study (15). Thus, it appears that the antagonism of discriminative stimulus in the rat could vary under different experimental conditions. This σ binding site may have some common structural feature with the dopamine receptor, because it binds haloperidol. However, this

Table 2. Regional distribution of multiple receptors in rat CNS

Region	Binding, dpm/mg of protein			
	σ (+)-[^{3}H]SKF 10,047*	δ [^{3}H][D-Ala^2,D-Leu^5]-Enkephalin†	μ [^{3}H]Naloxone‡	PCP [^{3}H]PCP§
Olfactory bulb	93 ± 6	295 ± 12	759 ± 11	
Frontal cortex	70 ± 4	198 ± 23	630 ± 20	641 ± 47
Olfactory tubercle	21 ± 17	615 ± 10	1,276 ± 23	
Nucleus accumbens	21 ± 1	403 ± 23	2,372 ± 65	
Septum	33 ± 17	102 ± 22	980 ± 119	
Caudate putamen	55 ± 4	530 ± 14	1,726 ± 24	
Remaining cortex	73 ± 6	266 ± 7	644 ± 8	665 ± 43
Globus pallidus	63 ± 9	270 ± 20	1,136 ± 76	
Amygdala	27 ± 18	117 ± 11	754 ± 17	694 ± 27
Hypothalamus	101 ± 9	188 ± 6	1,795 ± 56	
Anterior pituitary	90 ± 3	1 ± 6	124 ± 14	
Posterior pituitary	0 ± 2	20 ± 2	225 ± 5	
Hippocampus	112 ± 22	29 ± 11	251 ± 7	968 ± 75
Thalamus	70 ± 5	230 ± 14	1,267 ± 33	
Substantia nigra	18 ± 9	112 ± 38	1,739 ± 31	
Ventral tegmentum	97 ± 36	164 ± 6	1,943 ± 59	
Cerebellum	144 ± 11	37 ± 2	147 ± 18	359 ± 40
Pons and medulla	149 ± 7	162 ± 16	1,179 ± 104	358 ± 24
Spinal cord	191 ± 8	65 ± 6	820 ± 33	348 ± 20

Values are means ± SEM of at least two experiments done in triplicate.
*1.0 nM (+)-[^{3}H]SKF 10,047 in the presence of 1 μM naloxone and 100 mM NaCl. Naloxone at 1 μM had no effect. It was added to be consistent with the protocol for [^{3}H]EKC binding.
†0.3 nM [^{3}H][D-Ala^2,D-Leu^5]enkephalin.
‡0.5 nM [^{3}H]naloxone in 100 mM NaCl.
§5.0 nM [^{3}H]PCP in 100 mM NaCl.

σ binding site is not a dopamine receptor because it has opposite stereoselectivity for butaclamol compared with the dopamine receptor, and it does not bind clozapine or sulpiride. A comparative study between the σ and dopamine receptors must be undertaken.

The PCP receptor has been shown to bind PCP with higher affinity than SKF 10,047, EKC, and pentazocine (16). However, the affinity of SKF 10,047, EKC, and pentazocine for the σ binding sites reported here was much higher than that of PCP. SKF 10,047 (16) and EKC showed little stereoselectivity towards the PCP receptor but (+)-SKF 10,047 and (+)-EKC were highly stereoselective for the σ receptor. The regional distribution of the PCP receptor is similar to that reported previously (17, 18). The regional distribution of the PCP receptor is different than that of the σ binding sites. Therefore, it is not possible that the PCP and (+)-SKF 10,047 or (+)-EKC binding sites are different sites on the same receptor. These data suggested that the σ receptor and the PCP receptor may be different receptors with some similar properties.

Table 3. Relative potencies of drugs in inhibiting (±)-[^{3}H]EKC naloxone-inaccessible specific binding to rat brain and spinal cord membranes

Compound	IC_{50}, nM	
	Spinal cord*	Brain†
(+)-EKC	42 ± 5	53 ± 8
(−)-EKC	14,000 ± 5,100	11,000 ± 1,000
(+)-SKF 10,047	32 ± 8	38 ± 5
(−)-SKF 10,047	1,000 ± 80	1,000 ± 250
PCP	2,400 ± 1,200	1,700 ± 660

Binding was performed by using 5 nM (±)-[^{3}H]EKC in the presence of 1 μM naloxone and 100 mM NaCl. Values are means ± SEM.
*Three to six experiments in duplicate.
†Three experiments in quadruplicate.

There is *in vivo* evidence that supports the existence of a naloxone-inaccessible σ receptor. SKF 10,047 and cyclazocine generalized to PCP but the PCP-like discriminative effects of cyclazocine and SKF 10,047 in rats were not blocked by naloxone (19) or a relatively lower dose of naltrexone (20). The cyclazocine-like discriminative effects of SKF 10,047 in squirrel monkeys was also not blocked by naltrexone (21). Naloxone also could not block the SKF 10,047-induced increase in locomotor activity and agitation in rats (22). The PCP-like discriminative stimuli of (+)- and (−)-SKF 10,047 in rats were not blocked by naloxone (23).

Very recently, Su (24) reported evidence for an etorphine-inaccessible receptor in the guinea pig brain by using (±)-[^{3}H]SKF 10,047 as the radiolabeled ligand. His receptor shared a number of similarities with the σ receptor described here. However, the affinity of his receptor for active compounds is generally lower by 1/8th to 1/4th. This may be due to differences in experimental conditions. There is one major difference in that EKC bound the σ receptor reported here with fairly high affinity, whereas it bound the σ receptor reported by Su (24) with very low affinity. This difference could not be explained by either sodium ion effect or species difference between the rat and guinea pig, because (+)-[^{3}H]EKC and (+)-[^{3}H]SKF 10,047 also bind the same receptor in the guinea pig brain (unpublished data). T.-P. Su (personal communication) has recently confirmed that (+)-EKC also bound the σ receptor in the guinea pig brain with fairly high affinity.

The finding that opiates of the benzomorphan series bind the σ receptor may explain their psychotomimetic activities. However, no "σ-type" effect of EKC was observed in dogs (1) and rats (12). It is possible that the highly sedative effect of EKC could mask the observable σ-type behavioral responses in the animals. It is also possible that (+)-EKC is not a full agonist at the σ receptor. The finding that [^{3}H]EKC binds the σ binding sites suggests caution in using this ligand to label κ receptors.

Care should be taken to block the μ, δ, and σ receptors with unlabeled specific ligands for these receptors in order to reveal the κ receptors. The use of (−)-[^{3}H]EKC should eliminate binding to the σ binding sites.

It is interesting that the distribution of the σ binding sites in the anterior pituitary is relatively high, whereas it is very low for the μ and δ receptors. It remains to be determined whether the σ binding sites play any role in regulating hormonal release or synthesis in the anterior pituitary.

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