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[^{3}H]Ethylketocyclazocine Binding to NCB-20 Hybrid Neurotumor Cells

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SUMMARY

Ethylketocyclazocine (EKC) binds to two sites on NCB-20 neuroblastoma × Chinese hamster brain hybrid cells (K_{D_H} = 2 nM, B_{max} = 21,000 sites/cell; K_{D_L} = 27 nM, B_{max} = 140,000 sites/cell. The high-affinity site has been characterized as a δ opiate receptor. The low-affinity site is relatively benzomorphan-specific; opioid peptides, morphine, etorphine, and naloxone do not compete at it. Rank order of potency among benzomorphans is (+)-EKC > Mr 2267 > (+)-ketocyclazocine > (+)-SKF 10047 > bremazocine > cyclazocine. Among other drugs of interest that inhibit [^{3}H]EKC binding are phencyclidine and its analogues, K_i values for which are 0.2–40 μM. Stereoselectivity is the reverse of other opioid receptors: (+)-EKC ⋙ (−)-EKC, Mr 2267 > Mr 2266, (+)-SKF 10047 > (−)-SKF 10047. The site is sensitive to trypsin, but not to *N*-ethylmaleimide. Binding is insensitive to nucleotides, slightly sensitive to physiological concentrations of sodium, magnesium, and manganese ions and to EDTA but not EGTA.

INTRODUCTION

Benzomorphans, potent synthetic opioid analgesics, differ significantly from morphine and morphinans. Certain benzomorphans, including KC[2] and EKC, which are antinociceptive in dogs and rodents, neither precipitate withdrawal nor suppress abstinence in morphine-dependent dogs or monkeys (1–3). Martin and colleagues (3, 4) postulated three receptor types to account for differences between morphine and benzomorphans and among benzomorphans in the spinal dog. These were a μ receptor mediating analgesia and other effects associated with morphine, a κ receptor mediating analgesic and sedative effects associated with benzomorphans such as KC and EKC, and a σ receptor mediating psychoactive effects associated with benzomorphans such as SKF 10047 and cyclazocine.

Recent data from binding assays which suggest that benzomorphans are not as site-specific as they might appear from physiological studies nonetheless support the receptor distinctions made by Martin *et al.* (3, 4). Kosterlitz's group has described [^{3}H]EKC binding in guinea pig brain to an apparent κ site (5). Others have described similar binding in rat brain (6, 7). Zukin and Zukin (8), using [^{3}H]cyclazocine, have described an additional site in rat brain similar to a σ receptor. Recently, we reported benzomorphan binding in a neurotumor cell line that is not inhibited by morphine or enkephalins and which is unique among cell lines tested to NCB-20 (9). The purpose of this report is to resolve and characterize the [^{3}H]EKC binding.

MATERIALS AND METHODS

(+)-[^{3}H]EKC (15 Ci/mmole) was obtained from New England Nuclear Corporation. Drugs and their sources were as follows: DADLE, Leu-enkephalin and Met-enkephalin, Dr. S. Wilkinson, Burroughs-Wellcome; SKF 10047, cyclazocine, ketocyclazocine, cyclorphan, and phencyclidine and its analogues, Dr. R. S. Zukin, Albert Einstein College of Medicine; SKF 10047, EKC, UM 1071R and UM 1071S, and Mr 2266 and Mr 2267, Dr. J. H. Woods, University of Michigan; EKC enantiomers, WIN 44441-2 and WIN 44441-3, SKF 10047, Dr. W. Michne, Sterling Winthrop; morphine, etorphine, naloxone, pentazocine, levorphanol, dextrorphan, oxilorphan, diprenorphine, and codeine, Dr. B. Wainer, University of Chicago; verapamil and D600, Dr. R. Karl, University of Chicago; WB 4101, Dr. D. U'Prichard, Northwestern

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[2] The abbreviations used are: KC, ketocyclazocine; EKC, ethylketocyclazocine; SKF 10047, *N*-allylnorcyclazocine; DADLE, [D-Ala^2,D-Leu^5]enkephalin; Mr 2266 and Mr 2267, the (−)- and (+)-isomers, respectively, of 2-(3-furylmethyl)-5,9-diethyl-2′-hydroxy-6,7-benzomorphan; UM 1071R and UM 1071S (also known as Mr 2034 and Mr 2035), the (−)- and (+)-isomers, respectively, of 5,9-dimethyl-2′-hydroxy-2-tetrahydrofurfuryl-6,7-benzomorphan; WIN 44441-2 and WIN 44441-3, the 9*R* and *S* isomers, respectively, of 1-cyclopentyl-5-(2,5,9-trimethyl)-2′-hydroxy-6,7-benzomorphan-9-yl)-3-pentanone; PCP, phencyclidine; TCP, 1-[1-(2-thienyl)cyclohexyl]piperidine; *N*-EPC, *N*-ethyl-1-phenylcyclohexylamine; PCM, 1-(1-phenylcyclohexyl)morpholine; WB 4101, 2-[(2′,6′-dimethoxyphenoxyethyl)aminomethyl]-1,4-benzodioxane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; NEM, *N*-ethylmaleimide; Gpp(NH)p, guanyl-5′-yl-imidodiphosphate; BSA, bovine serum albumin.

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University; dynorphin, β-endorphin, Met-enkephalin [Arg^6,Phe^7], kyotorphin, and dermorphin, Peninsula Laboratories; bremazocine, Sandoz; norepinephrine, serotonin, dopamine, histamine, and carbachol, Sigma Chemical Company.

NCB-20 is a hybrid cell line derived from a cross between the N18TG2 mouse neuroblastoma and a Chinese hamster brain explant (10). Cells were grown at 37° in a modified Eagle's minimal essential medium (11) supplemented with 10% fetal calf serum and gentamycin sulfate (25 μg/ml) on 100-mm tissue culture dishes in a humidified atmosphere of 10% CO_2 and 90% air. Cells were harvested at confluency by scraping with a rubber policeman and centrifuged for 10 min at 50,000 × g. They were resuspended in ice-cold 50 mM Tris·HCl (the pH of which was 7.4 at 25°) for 30 min to swell them, then disrupted by Polytron at two-thirds maximal setting for 30 sec. Tissue was centrifuged for 10 min at 50,000 × g, resuspended, and centrifuged again. Pellets were stored at −80°.

For equilibrium binding assays, tissue was resuspended in 50 mM Tris·HCl (pH 7.4) and incubated with radiolabeled ligand for 45 min at 25° in a final volume of 0.5 ml. Points were assayed in triplicate for saturation binding and in duplicate for competition binding. At the end of the incubation period, 1 ml of ice-cold Tris was added to the incubation volume and this was immediately vacuum-filtered over Whatman GF/C filters, followed by two 5-ml washes with ice-cold Tris. For non-equilibrium assays, duplicate 1-ml samples were pipetted from a pooled 50-ml incubation volume directly onto filters, which were then washed twice. Filters, after air drying, were counted at 35% efficiency. Specific binding was calculated as indicated in the figure legends.

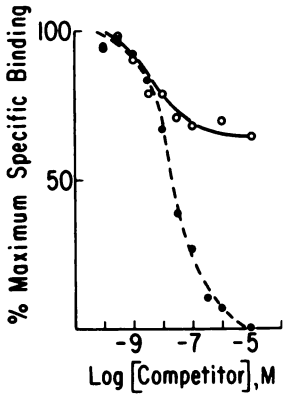

FIG. 1. *[^{3}H]EKC competition curves*
Increasing concentrations of EKC (●) and DADLE (○) were incubated with a fixed concentration of [^{3}H]EKC (7 nM). Control binding was 1500 cpm, of which 18% was nonspecific. Data are from a single experiment representative of four experiments.

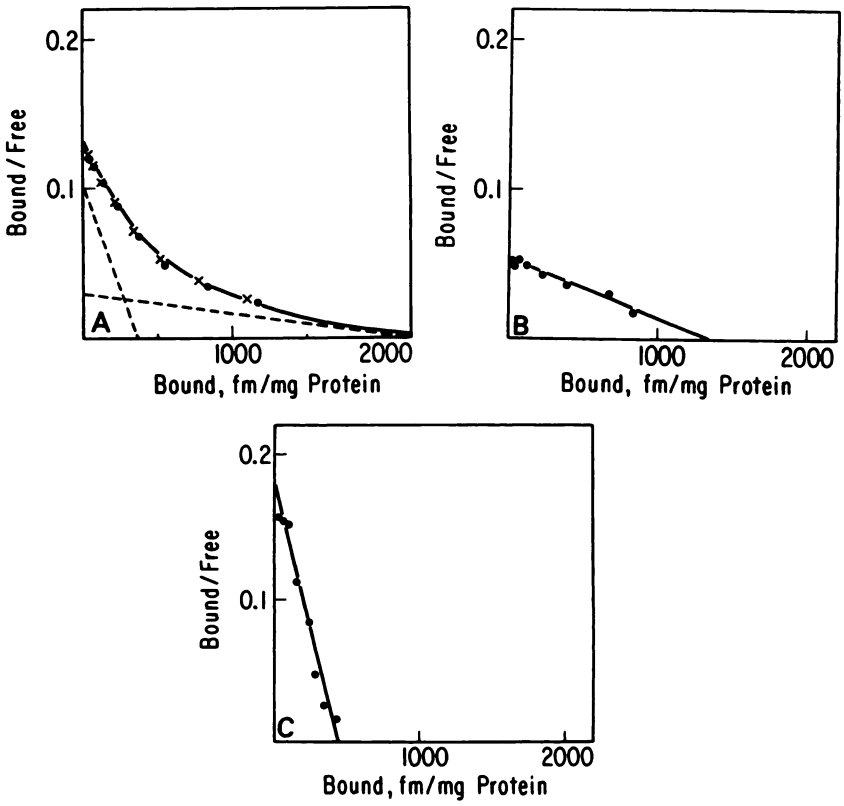

FIG. 2. *Scatchard analysis of [^{3}H]EKC binding*
Increasing concentrations of [^{3}H]EKC (0.2–40 nM) were incubated with membranes to equilibrium under the following conditions: A, [^{3}H]EKC with 10^{-5} M EKC blanks; protein = 0.35 mg/0.5 ml. B, [^{3}H]EKC with 10^{-5} M DADLE blanks; protein = 0.67 mg/0.5 ml. C, [^{3}H]EKC plus 10^{-5} M DADLE in totals and blanks with 10^{-5} M EKC blanks; protein = 0.37 mg/0.5 ml. Figures are representative experiments from a number indicated in the text. ●, Data point; ×, computer-generated point. To resolve components of curve A, successive approximations of K_{D_H}, K_{D_L}, B_{max_H}, and B_{max_L} are supplied, along with the free concentration, F, for the equation $B = [F \cdot B_{max_H}/(F + K_{D_H})] +]F \cdot B_{max_L}/(F + K_D)]$ where B = specifically bound ligand, until a line of good fit results.

Protein concentration was approximately 0.4 mg/0.5 ml for equilibrium assays and 0.4 mg/ml for non-equilibrium assays. Specific binding was a linear function of protein concentration over the range of protein concentrations in these experiments. Protein was assayed according to the method of Lowry *et al.* (12) with BSA as standard.

There was no significant filter binding or apparent degradation of [^{3}H]EKC in these assays. Thin-layer chromatography of [^{3}H]EKC in 9:1 chloroform/methanol on silica gel G yielded a single peak with R_F = 0.65. [^{3}H]EKC preincubated with tissue yielded the same profile.

RESULTS

Figure 1, competition binding of DADLE and EKC against [^{3}H]EKC, summarizes a singular difference between NCB-20s and other cell lines examined. A sizeable portion of [^{3}H]EKC binding in this cell line is inaccessible to DADLE, other opioid peptides, or morphinans. In other cell lines, DADLE and morphine displace all specific [^{3}H]EKC binding because all of it is to an enkephalin-sensitive δ site. Only a portion of [^{3}H]EKC binding in NCB-20s is to a δ site and thus accessible to DADLE (9).

Under different conditions, one can assay specific [^{3}H]EKC binding at either of the two sites on NCB-20s. Figure 2 illustrates a series of Scatchard plots of [^{3}H]EKC saturation binding. Specific binding, defined as the difference between [^{3}H]EKC bound in the absence and presence of 10^{-5} M EKC, yields a curvilinear Scatchard plot in Fig. 2A, indicative, as subsequent experiments confirm, of two binding sites. Computer-assisted approximations of binding parameters (mean ± standard error of the mean; n = 6) are K_{D_H} = 1.6 ± 0.4 nM, B_{max} = 280 ± 50 fmoles/mg of protein, K_{D_L} = 27 ± 6 nM, and B_{max_L} = 1840 ± 210 fmoles/mg of protein. With 8 × 10^6 cells/mg of membrane protein, this is equal to 21,000 high-affinity and 140,000 low-affinity [^{3}H]EKC binding sites/cell. When specific binding is defined in Fig. 2B as the difference in [^{3}H]EKC bound in the absence and presence of 10^{-5} M DADLE, binding is to the high-affinity site only. K_D and B_{max} (n = 3) are 4.7 ± 1.1 nM and 440 ± 115 fmoles/mg of protein. In Fig. 2C, specific binding is the difference between [^{3}H]EKC binding with 10^{-5} M DADLE in the absence and presence of 10^{-5} M EKC. These conditions block the high-affinity site altogether and direct [^{3}H]EKC binding to the lower-affinity site. K_D and

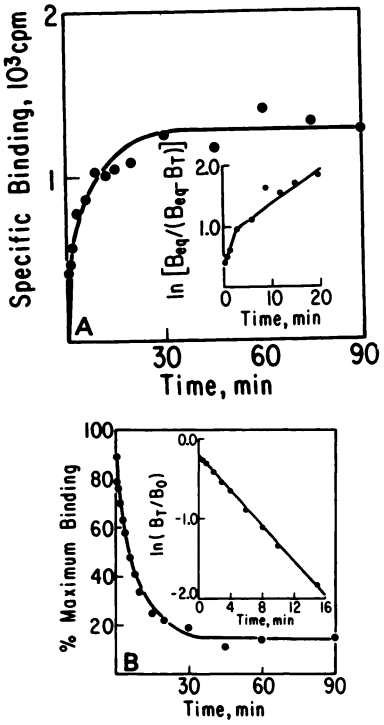

FIG. 3. *Kinetics of [^{3}H]EKC binding*
A, Time course of association of [^{3}H]EKC binding. The [^{3}H]EKC concentration was 7 nM. B, Time course of EKC dissociation. After [^{3}H]EKC binding had equilibrated (45 min), 10^{-5} M EKC was added. Figures show representative experiments from a number indicated in the text.

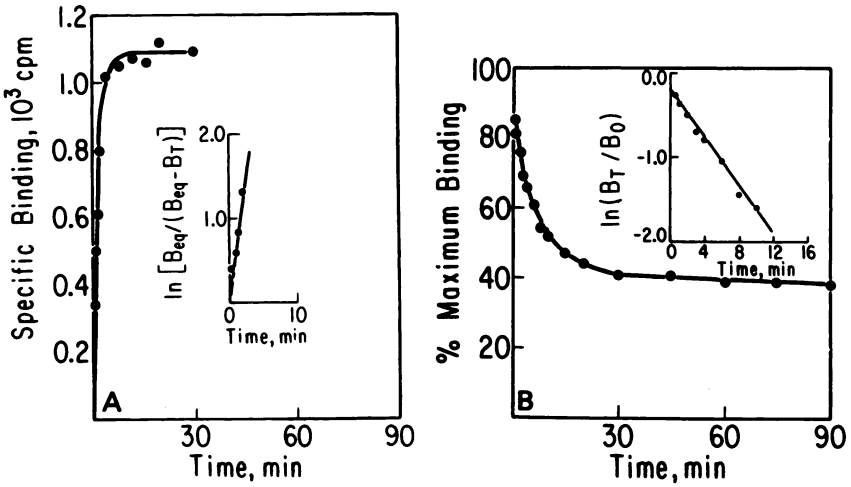

FIG. 4. *Kinetics of [^{3}H]EKC binding to the high-affinity site*
A, Time course of association of 1.2 nM [^{3}H]EKC. Specific binding is the difference between [^{3}H]EKC bound in the absence and presence of 10^{-5} M DADLE. The association rate constant is calculated as K_1 = (K_{obs} − K_{-1})/[L], where K_{obs} is the slope of the transform, K_{-1} is the dissociation rate constant determined as in B, and [L] is the [^{3}H]EKC concentration. Total steady-state binding is 15% of free ligand. B, Dissociation time course. After equilibration of [^{3}H]EKC binding, 10^{-3} M DADLE was added for a final concentration of 10^{-5} M DADLE. K_{-1} is equal to the negative slope of the transform. Figures show representative experiments from a number indicated in the text.

B_{max} under these conditions (n = 7) are 21 ± 2 nM and 1450 ± 170 fmoles/mg of protein. Results with these various conditions make clear that the high-affinity EKC site is the δ site previously described and the low-affinity site is an as yet unclassified site which can be analyzed separately.

Non-equilibrium assays essentially confirm the results of saturation binding assays. Figure 3A represents a time course of association of [^{3}H]EKC done, like subsequent ones, under conditions that allow pseudo-first order kinetic analysis. The linear transform of these data discloses two components reflecting different rates of association of [^{3}H]EKC with the two sites. This would appear to account for most of the difference in the affinity of the two sites, since the time course of dissociation transformed as in Fig. 3B yields a straight line virtually for its entirety, indicative of a uniform rate of dissociation.

Again analyzing binding to a single site, one can determine association and dissociation rates for each site. In Fig. 4A and B, binding is directed to the high-affinity site. The K_1 is 0.25 ± 0.06 nM^{-1} min^{-1} (n = 2) and the K_{-1} is 0.13 ± 0.019 min^{-1} (n = 2). The K_D determined from these rates is equal to 0.5 nM, in good agreement with the computer-assisted approximation in Fig. 2A but somewhat higher affinity than the equilibrium K_D under the same conditions in Fig. 2B. In Fig. 5A and B binding is directed toward the low-affinity site. The K_1 is 0.0052 ± 0.0008 nM^{-1} min^{-1} (n = 2) and the K_{-1} is 0.071 ± 0.013 min^{-1} (n = 3). These yield a K_D of 14 nM, in good agreement with equilibrium determinations of Fig. 2A and C. From these experiments, it appears that a slower rate of association of [^{3}H]EKC with the lower-affinity site accounts for the lower affinity. The dissociation rate is not substantially different, actually being a little slower from the low-affinity site than the high-affinity site.

We have assayed series of compounds in competition for the low-affinity [^{3}H]EKC site. Table 1 is a pharmacological profile of the site. Only benzomorphans compete with very high affinity. Among those that compete, the rank order of enantiomers is the reverse of that at other opioid binding sites. While (+)-EKC is the most potent compound tested, (−)-EKC hardly competes. Dextrorphan competes with low affinity, and levorphanol hardly competes. Among other enantiomeric pairs, there is a varying degree of selectivity: Mr 2267 ≫ Mr 2266, (+)-SKF 10047 ≫ (−)-SKF 10047, UM 1071S ± UM 1071R, WIN 44441-2 ≫ WIN 44441-3. The other class of drugs that compete for this site comprises dissociative anesthetics, PCP and its analogues: TCP > *N*-EPC, PCP > PCM > ketamine. Although affinities in this class are not very high (0.2–40 μM), they are close to those reported for phencyclidine interactions with other systems (14–18). Substances with which μ or δ opiate receptors are identified, including morphine, naloxone and enkephalins, do not compete for binding at this site. Verapamil and D600 both compete for binding, but another very potent calcium channel blocker, nifedipine, does not. None of the biogenic amines tested competed for binding. Verapamil, in addition to blocking calcium channels, has been reported to inhibit the reuptake of some biogenic amines (19). It appears from our results that this site is neither a calcium channel nor a reuptake site for any of these transmitters, as well as a receptor for any of them.

One might suppose that a physiologically significant binding site would be a protein, although this is not always the case (e.g., choleragen and GM_1 ganglioside). Trypsin treatment destroys binding to this low-affinity site in a dose-dependent manner (Fig. 6). The degree of reduction of binding at this site is similar to that is seen at a δ site (20). NEM is an alkylating reagent that reacts with the sulfhydryl groups of cysteine residues in proteins. It has been used to inactivate other benzomorphan binding sites (5). While this site is trypsin-sensitive, it is not sensitive to NEM. Dithiothreitol, a reducing agent, will reduce (among other things) disulfide bonds between cystine residues. It also has no effect on binding.

Physiological concentrations of a number of ions affect ligand binding. Sodium, in particular, reduces the binding of opiate agonists (21). Guanine nucleotides reduce binding of agonists to some opiate receptors (22). We have screened a number of salts in Tris·acetate and Tris·HCl buffers. Neither anions nor buffer type affected the

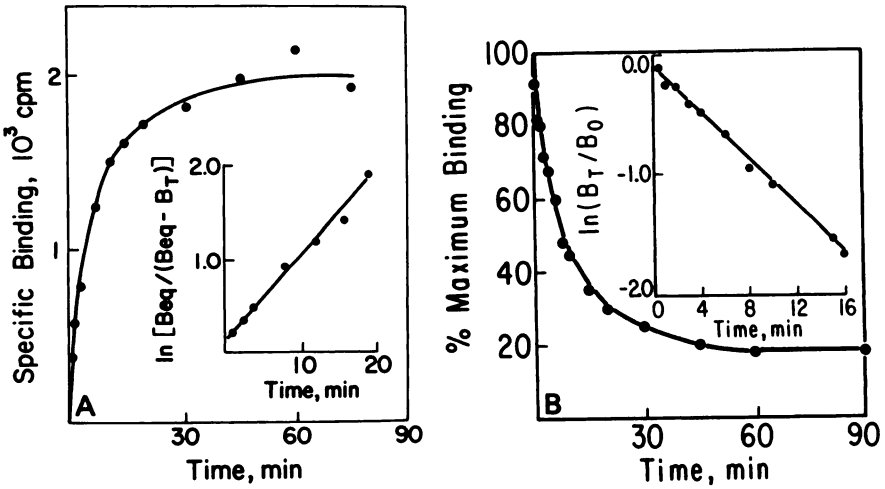

FIG. 5. *Kinetics of [^{3}H]EKC binding to the low-affinity site*

A, Time course of association of 7 nM [^{3}H]EKC binding. Specific binding is the difference between [^{3}H]EKC plus 10^{-5} M DADLE in the absence and presence of 10^{-5} M EKC. The association rate constant is calculated as in Fig. 4A. Total steady-state binding is 5% of free ligand. B, Dissociation time course. After equilibration of [^{3}H]EKC binding in the presence of 10^{-5} M DADLE, 10^{-5} M EKC was added. Figures show representative experiments from a number indicated in the text.

TABLE 1

Rank order of potencies against [^{3}H]EKC/DADLE binding

Increasing concentrations of drugs were incubated with a fixed concentration of [^{3}H]EKC (7 nM) in the presence of 10^{-5} M DADLE. IC_{50} values were determined by log-probit analysis. K_i values were determined according to the formula $K_i = IC_{50}/(1 + K_D/[L])$ (13). Values are means ± standard error of the mean of two experiments. Other substances tested at 10^{-4} M that did not inhibit binding include DADLE, Leu-enkephalin, Met-enkephalin, Met-enkephalin-[Arg^6, Phe^7], dynorphin, morphine, etorphine, naloxone, diprenorphine, oxilorphan, codeine, kyotorphin, apomorphine, norepinephrine, serotonin, dopamine, histamine, carbachol, and nifedipine. Peptide assays contained 0.1% BSA. Norepinephrine, serotonin, and dopamine assays contained 10^{-5} M pargyline and 0.01% ascorbic acid.

Competitor	K_i ± SEM
	nM
(+)-EKC	3 ± 0
Mr 2267	6 ± 1
(±)-KC	9 ± 2
(+)-SKF 10047	13 ± 4
Bremazocine	63 ± 4
Cyclazocine	82 ± 4
Pentazocine	150 ± 60
TCP	210 ± 100
UM 1071S	300 ± 90
UM 1071R	330 ± 130
WB 4101	360 ± 260
Dextrorphan	450 ± 30
Mr 2266	480 ± 180
Cyclorphan	1,100 ± 100
(−)-SKF 10047	1,200 ± 470
Etazocine	1,500 ± 700
N-EPC	1,900 ± 400
L-Verapamil	2,500 ± 800
PCP	2,700 ± 670
D600	6,000 ± 2,700
PCM	9,500 ± 2,500
Nalorphine	12,000 ± 3,000
WIN 44,441-2	12,000 ± 7,700
WIN 44,441-3	27,000 ± 10,000
Ketamine	41,000 ± 0
D-Verapamil	>10^{-5} M
(−)-EKC	>10^{-5} M
β-Endorphin	>10^{-5} M
Dermorphin	>10^{-4} M
Levorphanol	>10^{-4} M

response to cations. Table 2 is a summary of the effects of a number of cations and nucleotides on binding. The effects are, at most, slight. Sodium increased binding to the same extent that magnesium and manganese reduced it. The effect of the latter two, although slight, appears specific since calcium had no effect and EDTA but not EGTA had an opposite effect. Guanine nucleotides did not affect binding at all.

DISCUSSION

In an earlier report, we described [^{3}H]EKC binding to two sites on NCB20 cells (11). The lower-affinity site, unique to NCB20s among cell lines studied, we suggested was a κ receptor. Another radiolabeled benzomorphan, [^{3}H]SKF 10047, showed saturation binding similar to

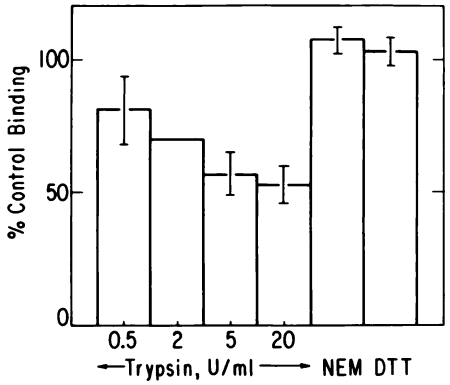

FIG. 6. *Sensitivity of the low-affinity site to protein-modifying reagents*

Membranes were incubated with reagents for 30 min at 25°. Concentrations of NEM and dithiothreitol (*DTT*) were 1 mM. Tissue was pelleted and resuspended for assay of specific binding. Error bars represent standard error of the mean of two to four determinations.

that of [^{3}H]EKC; Scatchard analysis disclosed multiple [^{3}H]SKF 10047 binding sites and a total site number approximately equal to the number of [^{3}H]EKC binding sites, yet in competition binding assays EKC displaced less [^{3}H]SKF 10047 binding than did SKF 10047. Based on this apparent difference in efficacy, we suggested the possibility of an additional [^{3}H]SKF 10047 binding site, perhaps a σ receptor.

The experiments detailed in this report permit discrete characterization of the low-affinity [^{3}H]EKC binding site with the high-affinity site blocked. Similar experiments using [^{3}H]SKF 10047 as labeled ligand and blocking EKC binding sites with 10^{-5} M EKC disclose no additional saturable [^{3}H]SKF 10047 binding. The difference in the efficacy of EKC and SKF 10047 as displacers of [^{3}H]SKF 10047 binding that was originally observed was most likely due to displacement of nonspecifically bound [^{3}H]SKF 10047 with high concentrations of SKF 10047. Thus, both [^{3}H]EKC and [^{3}H]SKF 10047 both bind to two classes of sites on NCB20s, a high-affinity, enkephalin-

TABLE 2

[^{3}H]EKC/DADLE binding: effects of ions and nucleotides

The binding of 7 nM [^{3}H]EKC plus 10^{-5} M DADLE in the absence and presence of 10^{-5} M EKC was assayed with the indicated substances. Values are mean ± standard error of the mean from three experiments except for Gpp(NH)p, which was assayed once. Control specific binding was approximately 1200 cpm.

Treatment	% Control binding
NaCl, 100 mM	113 ± 2
KCl, 1 mM	100 ± 8
$CaCl_2$, 1 mM	98 ± 1
$MgCl_2$, 1 mM	91 ± 2
$MnCl_2$, 1 mM	90 ± 1
EDTA, 1 mM	118 ± 2
EGTA, 1 mM	103 ± 1
Gpp(NH)p, 500 μM	103
GTP, 500 μM	96 ± 1
GDP, 500 μM	96 ± 2
GMP, 500 μM	96 ± 2
ATP, 500 μM	101 ± 2

sensitive δ site, and a lower-affinity, relatively benzomorphan-specific site.

Despite labeling of the low-affinity site with [^{3}H]EKC, in view of the many other compounds which do not inhibit binding, the site is unlike what has been described as a κ receptor. Dynorphin, which appears to be an endogenous ligand for the κ receptor (23, 24), does not inhibit binding to the low-affinity site in NCB20s. Etorphine and naloxone compete with high affinity for what has been described as a κ site in guinea pig brain, whereas morphine and enkephalins compete with low affinity (5). None of these compounds binds to the low-affinity [^{3}H]EKC site in NCB20s. Using [^{3}H]diprenorphine, Chang *et al.* (6) have described a benzomorphan binding site in rat brain distinct from μ and δ receptors. In its affinity for naloxone, oxilorphan, and diprenorphine, this site can be distinguished from the low-affinity benzomorphan binding site in NCB20s.

Since the σ receptor was postulated to account for the psychoactive effects of SKF 10047 and cyclazocine (3), the affinity of PCP and its analogues (potent psychotomimetics) for this site raises the possibility that the site corresponds to a σ receptor. In its drug specificity, the site is similar to a [^{3}H]cyclazocine binding site described in rat brain membranes (8) and a [^{3}H]PCP binding site described in rat brain membranes and olfactory bulb slices (25). Notwithstanding the general similarity of the low affinity [^{3}H]EKC site in NCB20s to the [^{3}H]cyclazocine and [^{3}H]PCP site or sites in brain, there are specific differences. In particular, although the affinity for PCP is nearly equal in brain and cell membranes, the affinity for benzomorphans is generally 2 orders of magnitude greater in cells than in brain. Additionally, the reverse stereoselectivity for benzomorphans characteristic of the site in NCB20s does not extend to the site in brain (26). It would appear then that despite gross similarities, these are different sites.

Drug discrimination studies present evidence for the occurrence of a site of common interaction for PCP and benzomorphans, but its stereoselectivity is unclear. Rats trained to discriminate cyclazocine generalize to KC, SKF 10047, EKC, PCP, and ketamine, but not morphine (27). Discrimination is only partially blocked by naltrexone. Trained on PCP, rats generalize to ketamine, cyclazocine, and dextrorphan (28). From these and a series of other drug discrimination studies, it is clear that some benzomorphan cues which are not common to morphine and are not antagonized by naloxone or naltrexone are common to PCP. In these respects, behavioral data are similar to binding data in brain and in the cells.

With respect to the stereoselectivity of the site involved, the evidence is equivocal. In one study, PCP generalized to (+)- but not (−)-SKF 10047 (29). In another similar study, PCP generalized to (−)-cyclazocine at one-tenth the dosage required for generalization to (+)-cyclazocine (30). Although behavioral and electrophysiological studies indicate that the (+)-isomer of a PCP analogue is more potent than the (−)-isomer (31, 32), this order of stereoselectivity would not necessarily apply to other classes of drugs. Because dextrorphan inhibits binding to the low-affinity [^{3}H]benzomorphan site in NCB20s and PCP generalizes to dextrorphan in behavioral studies, it would be of interest to know whether dextrorphan inhibits binding to the [^{3}H]cyclazocine/[^{3}H]PCP site in rat brain.

Another drug of potential interest in drug discrimination studies and binding studies in brain is the *alpha*$_1$-adrenergic antagonist WB 4101. The drug competes for low-affinity [^{3}H]EKC binding in NCB20s with an affinity (K_i = 330 nM) as great as or greater than that of PCP and its analogues. The drug has no psychoactive effects and is generally not associated with benzomorphans or PCP. It would be of interest to know whether WB 4101 inhibits binding of [^{3}H]cyclazocine and [^{3}H]PCP in rat brain membranes and whether it is, perhaps, an antagonist of these psychoactive substances in drug discrimination studies.

A problem with the putative σ receptor is that there is no specific σ receptor bioassay. Hence, beyond psychotomimetic effects related to it, the σ receptor is a vague entity. Once a cellular response is associated with the low-affinity [^{3}H]benzomorphan binding site in NCB20s, the cells may be a useful system for studying a physiological mechanism of benzomorphan action, possibly through a σ receptor.

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