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Characterization of [^{3}H][2-D-penicillamine, 5-D-penicillamine]-enkephalin binding to δ opiate receptors in the rat brain and neuroblastoma–glioma hybrid cell line (NG 108-15)

KAZUFUMI AKIYAMA*, KELVIN W. GEE*, HENRY I. MOSBERG†, VICTOR J. HRUBY‡, AND HENRY I. YAMAMURA*§

Departments of *Pharmacology and ‡Chemistry, University of Arizona, Tucson, AZ 85724; and †College of Pharmacy, University of Michigan, Ann Arbor, MI 48109

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ABSTRACT **Specific binding properties of the tritium-labeled δ opiate receptor agonist [^{3}H][2-D-penicillamine, 5-D-penicillamine]enkephalin ([^{3}H][D-Pen^2, D-Pen^5]enkephalin) were characterized in the rat brain and in a mouse neuroblastoma–rat glioma hybrid cell line (NG 108-15). Saturation isotherms of [^{3}H][D-Pen^2, D-Pen^5]enkephalin binding to rat brain and NG 108-15 membranes gave apparent K_d values of 1–6 nM. These values are in good agreement with the K_d value obtained from the kinetic studies. The B_{max} value in NG 108-15 membranes was 235.3 fmol/mg of protein. An apparent regional distribution of [^{3}H][D-Pen^2, D-Pen^5]enkephalin binding was observed in the rat brain. A number of enkephalin analogues inhibited [^{3}H][D-Pen^2, D-Pen^5]enkephalin binding with high affinity (IC_{50} values of 0.5–5.0 nM) in both NG 108-15 and rat brain membranes. However, putative μ receptor-selective ligands such as morphine, [D-Ala^2, MePhe^4, Gly^5-ol]enkephalin, [MePhe^3, D-Pro^4]morphiceptin, and naloxone were less effective inhibitors of [^{3}H][D-Pen^2, D-Pen^5]enkephalin binding in both systems tested. These data suggest that [^{3}H][D-Pen^2, D-Pen^5]enkephalin is a potent and selective ligand for the δ opioid receptor.**

It is well documented that the endogenous opioid pentapeptides [Met^5]enkephalin (H-Tyr-Gly-Gly-Phe-Met-OH) and [Leu^5]enkephalin (H-Tyr-Gly-Gly-Phe-Leu-OH), interact with several subtypes of opiate receptors that mediate different biological responses (1–3). A search for an opiate receptor ligand with a high degree of selectivity for one subtype of opiate receptor has been, therefore, essential to the elucidation of the function of opiate receptor subtypes.

A pioneering approach to the design of a ligand selective for the δ opioid receptor was implemented by Mosberg *et al.* (4), who demonstrated that the cyclic conformationally constrained enkephalin analogues [D-Pen^2, L-Cys^5]enkephalinamide and [D-Pen^2, D-Cys^5]enkephalinamide displayed substantial δ opioid receptor selectivity. Although the corresponding COOH-terminal analogues [D-Pen^2, L-Cys^5]-enkephalin and [D-Pen^2, D-Cys^5]enkephalin were found to possess even more pronounced δ opioid receptor selectivity (5), the same group recently found that further rigidity imposed by two *gem*-dialkyl substituents incorporated into the medium rings of the cyclic penicillamine (Pen; β,β-dimethylcysteine)-disubstituted enkephalin analogues H-Tyr-D-Pen-Gly-Phe-L-Pen-OH ([D-Pen^2, L-Pen^5]enkephalin) and H-Tyr-D-Pen-Gly-Phe-D-Pen-OH ([D-Pen^2, D-Pen^5]enkephalin) resulted in additional improvement in δ receptor selectivity. The selectivity of these analogues for the δ opioid receptor was greater than that of any other synthetic enkephalin developed thus far (6–8). Based upon pharmacological determinations using two bioassays (the mouse vas deferens and guinea pig ileum) and the rat brain radioreceptor assays (6, 7), [D-Pen^2, L-Pen^5]enkephalin and [D-Pen^2, D-Pen^5]enkephalin displayed greater selectivity for the δ opioid receptor than did the previously reported δ-selective opioid agonists [D-Thr^2, Leu^5, Thr^6]enkephalin (9) or [D-Ser^2, Leu^5, Thr^6]enkephalin (10).

The aforementioned evidence has led us to label [D-Pen^2, D-Pen^5]enkephalin with tritium in an attempt to determine the feasibility of using this radioligand to label δ opioid receptors directly. In the present study, we report the initial characterization of [^{3}H][D-Pen^2, D-Pen^5]enkephalin binding to the rat brain and NG 108-15 (a mouse neuroblastoma–rat glioma hybrid) membranes. NG 108-15 cells are thought to have only δ opioid receptors (3); thus, a comparison was made between δ opioid receptors in the two preparations. The data presented in this study clearly show that [^{3}H][D-Pen^2, D-Pen^5]enkephalin is potent and more selective for the δ opioid receptor than is the conventionally used [^{3}H][D-Ala^2, D-Leu^5]enkephalin.

MATERIALS AND METHODS

[D-Pen^2, D-Pen^5]Enkephalin, [D-Pen^2, L-Pen^5]enkephalin, [D-Pen^2, L-Cys^5]enkephalin, and [D-Thr^2, Leu^5, Thr^6]enkephalin were synthesized by solid-phase methods and purified by partition and gel chromatography by previously described methods (4–6). [D-Ala^2, D-Leu^5]Enkephalin and naloxone hydrochloride were purchased from Sigma and DuPont, respectively.

Tyr-D-Ala-Gly-MePhe-Gly-ol (D-Ala^2, MePhe^4, Gly^5-ol]enkephalin and [MePhe^3, D-Pro^4]morphiceptin were obtained from Peninsula Laboratories and K. J. Chang (Burroughs Wellcome, Research Triangle Park, NC), respectively. Morphine sulfate was obtained from T. F. Burks at the University of Arizona (Tucson, AZ). [^{3}H][D-Pen^2, D-Pen^5]-enkephalin (specific activity, 40 Ci/mmol; 1 Ci = 37 GBq) was custom-synthesized and generously provided by Amersham. [^{3}H][D-Ala^2, D-Leu^5]Enkephalin (43.6 Ci/mmol) and [^{3}H]naloxone (42.3 Ci/mmol) were purchased from New England Nuclear. All other chemicals were obtained from commercial sources.

Tissue Culture Techniques. NG 108-15 cells of low passage number were grown in the same manner as described (11). The cells were harvested and immediately frozen in liquid nitrogen and stored at −80°C.

Radioligand Binding Assays. NG 108-15 membranes were prepared by thawing the frozen cells in 50 mM Tris·HCl buffer (pH 7.4) at 25°C and homogenizing with a Polytron

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Abbreviation: Pen, penicillamine.

§To whom reprint requests should be addressed.

homogenizer. The membranes were washed twice in the same buffer by centrifugation at 48,000 × *g* for 10 min. The membranes were incubated at 25°C for 30 min between washes to facilitate removal of endogenous opioid-like factors. Twice-washed rat brain membranes (whole brain less cerebellum or discrete brain regions) were prepared as described (4–6). [^{3}H][D-Pen^2, D-Pen^5]Enkephalin (0.1–20 nM) was incubated with either NG 108-15 or rat brain membranes in 50 mM Tris·HCl buffer (pH 7.4) at 25°C supplemented with bovine serum albumin (1 mg/ml) and bacitracin (100 μg/ml). [^{3}H]Naloxone (1 nM) was incubated with rat brain membranes in the same buffer at 25°C. Incubation volumes were either 1 ml for NG 108-15 or 2 ml for rat brain membranes. In routine binding assays, incubations were performed under steady-state conditions. Incubations were maintained at 25°C for 9 hr for NG 108-15 membranes or 3 hr for rat brain membranes. When [^{3}H]naloxone was used, incubations were carried out at 25°C for 1 hr. Nonspecific binding was defined as binding in the presence of 1 μM unlabeled [D-Pen^2, D-Pen^5]enkephalin for [^{3}H][D-Pen^2, D-Pen^5]enkephalin or 1 μM naltrexone in the case of [^{3}H]naloxone binding assays. In some studies [^{3}H][D-Ala^2, D-Leu^5]enkephalin was investigated in parallel with [^{3}H][D-Pen^2, D-Pen^5]enkephalin under the same incubation conditions. All assays were carried out in duplicate on three to five determinations. The reactions were terminated by rapid filtration through Whatman GF/B glass-fiber filters. The filters were rinsed three times each with 5 ml of ice-cold 50 mM Tris·HCl buffer (pH 7.4). Radioactivity was extracted in 6-ml of scintillation mixture composed of 16 g of Omnifluor, 1 liter of Triton X-100, and 2 liters of toluene and was quantitated by liquid scintillation spectrophotometry.

Saturation and inhibition data were analyzed by using nonlinear least-squares regression analyses programs prepared for the Apple II Plus microcomputer (S.H.M. Research, Tucson, AZ). Protein concentrations were determined by the method of Lowry *et al.* with bovine serum albumin as a standard (12).

RESULTS

The effects of different incubation temperatures on specific [^{3}H][D-Pen^2, D-Pen^5]enkephalin binding were initially examined in rat brain membranes. Since steady-state specific binding at 0°C was much less than that at 25°C (data not shown), 25°C was used for all subsequent binding experiments.

Specific binding of [^{3}H][D-Pen^2, D-Pen^5]enkephalin in NG 108-15 membranes showed a linear relationship (correlation coefficient = 0.999) with protein concentrations up to 650 μg per assay. For routine assays, ≈90–220 μg of protein (5 × 10^5 cells) was used per assay tube. Tissue linearity also was observed in the rat brain membranes with concentrations up to 0.75 mg of protein per assay (data not shown). A 100-μl aliquot of a 10% brain homogenate (0.5 mg of protein) was routinely used in each assay.

Kinetic experiments were performed to determine association (k_{+1}) and dissociation (k_{-1}) rate constants. Specific [^{3}H][D-Pen^2, D-Pen^5]enkephalin (0.6 nM) binding to NG 108-15 membranes reached steady state only after 8–9 hr of incubation at 25°C (Fig. 1). Three hours of incubation at 25°C allowed the specific binding of 1 nM [^{3}H][D-Pen^2, D-Pen^5]enkephalin to reach steady state in rat brain membranes (data not shown). Since only a small fraction (about 2%) of the total concentration of the ligand ($[L]_T$) was bound even at steady state, association of specific [^{3}H][D-Pen^2, D-Pen^5]enkephalin binding to NG 108-15 membranes was plotted according to the pseudo-first-order equation (13):

$$\ln \frac{[LR]_e}{[LR]_e - [LR]} = k_{+1} t \frac{[L]_T [R]_T}{[LR]_e},$$

in which $[LR]_e$ and $[LR]$ are the concentrations of ligand–receptor complex at steady state and at time t during incubation, respectively and $[R]_T$ is the total concentration of binding sites. Linear regression (correlation coefficient = 0.999) of

$$\ln \frac{[LR]_e}{[LR]_e - [LR]}$$

versus time t generated a k_{+1} value of 1.87 × 10^8 $M^{-1} \cdot hr^{-1}$ (Fig. 1 *Inset*).

Dissociation of specifically bound [^{3}H][D-Pen^2, D-Pen^5]-enkephalin (0.6 nM) in NG 108-15 membranes was initiated

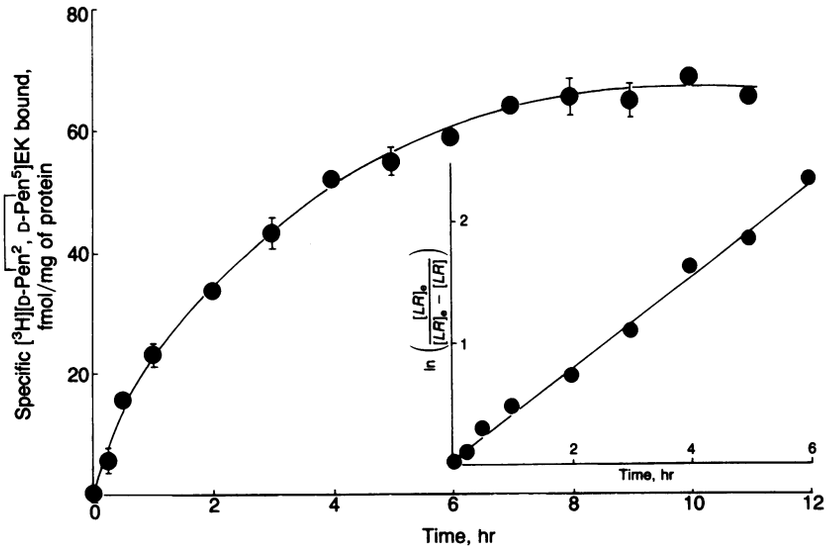

FIG. 1. Association of enkephalin (EK) analog [^{3}H][D-Pen^2, D-Pen^5]EK binding to NG 108-15 membranes. [^{3}H][D-Pen^2, D-Pen^5]EK (0.6 nM) was incubated with NG 108-15 membranes (0.110–0.138 mg of protein per assay) at 25°C for 0.25–11 hr. ●, Mean ± SEM of specific binding from four independent experiments. Values of nonspecific binding were stable (12.4–18.0 fmol/mg of protein) for up to 16 hr of incubation (data not shown). (*Inset*) Plot of the association of averaged specific binding of four experiments calculated according to the pseudo-first-order equation as shown in text. k_{+1} = 1.87 × 10^8 $M^{-1} \cdot hr^{-1}$.

by the addition of unlabeled [D-Pen^2, D-Pen^5]enkephalin (final concentration of 1 μM) after 9 hr of incubation (steady state). Subsequent incubations in the presence of 1 μM [D-Pen^2, D-Pen^5]enkephalin at various durations (0.25–7 hr) were carried out. During this incubation period, control specific binding (in the absence of 1 μM [D-Pen^2, D-Pen^5]enkephalin) showed no deterioration. Dissociation was plotted (Fig. 2) according to the first-order equation (13):

$$\ln (LR)/(LR)e = -k_{-1}t.$$

When a semi-logarithmic scale was used (Fig. 2 *Inset*), the plot linearly regressed (correlation coefficient = 0.997) and k_{-1} was calculated from $t_{1/2}$ = 3.45 hr:

$$k_{-1} = \frac{\ln 1/2}{3.45} = 0.20 \text{ hr}^{-1}.$$

From the values of k_{+1} and k_{-1}, the K_d was calculated to be 1.07 nM. It was difficult to perform dissociation studies in rat brain membranes because specific binding rapidly declined 5–6 hr after the initiation of the incubation.

Fig. 3 *A* and *B* depict computer-assisted best fits of parallel saturation isotherms of specific [^{3}H][D-Pen^2, D-Pen^5]enkephalin and [^{3}H][D-Ala^2, D-Leu^5]enkephalin binding in NG 108-15 membranes, respectively. The apparent dissociation constant (K_d) and the maximal number of binding sites (B_{max}) for [^{3}H][D-Pen^2, D-Pen^5]enkephalin are almost identical to those for [^{3}H][D-Ala^2, D-Leu^5]enkephalin. The K_d (1.24 nM) for [^{3}H][D-Pen^2, D-Pen^5]enkephalin from saturation studies was in a good agreement with the K_d derived from the kinetic studies (k_{-1}/k_{+1} = 1.07 nM). The Hill slopes for [^{3}H][D-Pen^2, D-Pen^5]enkephalin and [^{3}H][D-Ala^2, D-Leu^5]enkephalin binding were 1.10 (range, 1.06–1.16), and 0.96 (range, 0.93–1.35), respectively.

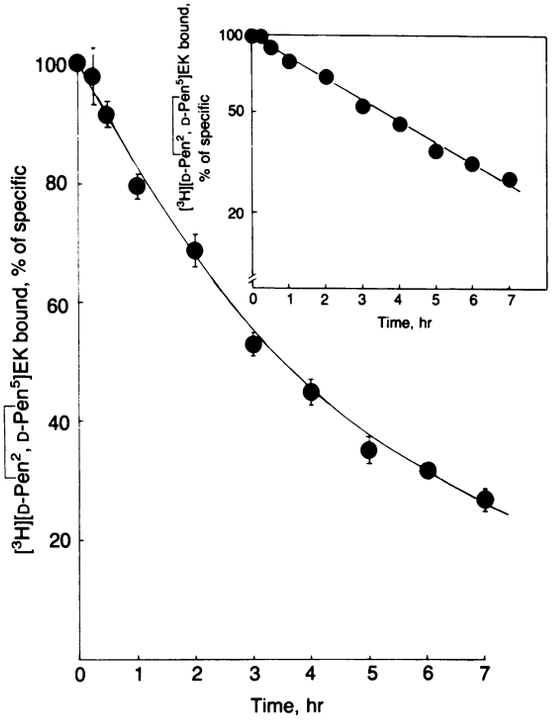

FIG. 2. Dissociation of enkephalin (EK) analog [^{3}H][D-Pen^2, D-Pen^5]EK binding from NG 108-15 membranes. Specific [^{3}H][D-Pen^2, D-Pen^5]EK (0.6 nM) binding to NG 108-15 membranes was equilibrated (9 hr of incubation at 25°C) before the initiation of the ligand dissociation by the addition of [D-Pen^2, D-Pen^5]EK (final concentration, 1 μM) to the reaction medium. Incubations were terminated at various times (0.25–7 hr) after equilibrium by rapid filtration. Mean ± SEM from five independent experiments. (*Inset*) Dissociation of the ligand was plotted according to the first-order equation on a semi-logarithmic scale as shown in the text. k_{-1} = 0.2 hr^{-1}.

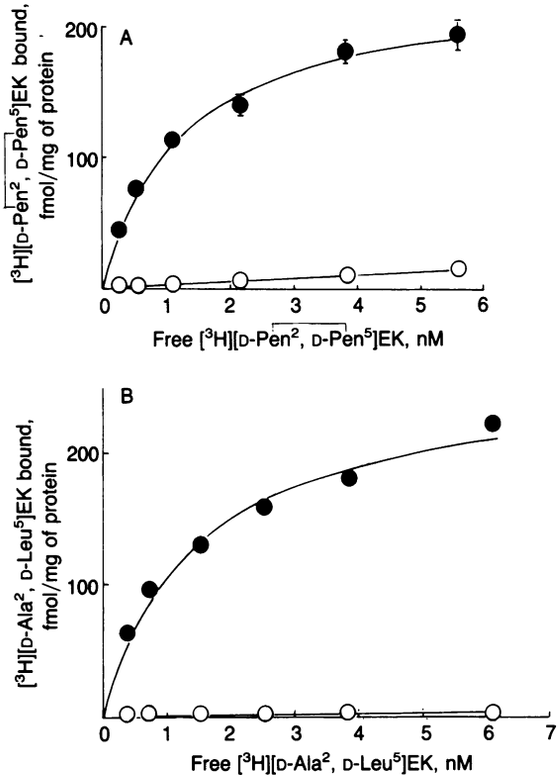

FIG. 3. Parallel saturation isotherms of enkephalin (EK) analogs [^{3}H][D-Pen^2, D-Pen^5]EK (*A*) and [^{3}H][D-Ala^2, D-Leu^5]EK (*B*) binding to NG 108-15 membranes. Saturation isotherms of both ligands were derived from the same cell preparations. Incubations were performed in the same manner as described in the text. Nonspecific binding was defined as binding in the presence of 1 μM unlabeled [D-Pen^2, D-Pen^5]EK. ●, Mean ± SEM of specific binding from three independent experiments. The averaged data were weighted (inverse of coefficient of variation) for the best fit curve. ○, Mean ± SEM of nonspecific binding. The following values were obtained: B_{max} = 235 ± 13 fmol/mg of protein (arithmetic mean ± SEM) and K_d = 1.24 nM ± 0.06 nM (geometric mean ± SEM) for [^{3}H][D-Pen^2, D-Pen^5]EK; B_{max} = 259 ± 6 fmol/mg of protein and K_d = 1.40 ± 0.11 nM for [^{3}H][D-Ala^2, D-Leu^5]EK.

Table 1. [^{3}H][D-Pen^2, D-Pen^5]Enkephalin binding to membranes from NG 108-15 cells and various rat brain regions

	K_d,* nM	B_{max},† fmol/mg of protein
NG 108-15	1.24 ± 0.06	235.3 ± 13.1
Rat brain region		
Cerebral cortex	3.3 ± 0.2	117.0 ± 3.0
Hippocampus	4.4 ± 0.8	64.6 ± 3.6
Pons-medulla	3.3 ± 0.4	39.0 ± 6.5
Cerebellum	5.2 ± 1.7	32.2 ± 6.6

[^{3}H][D-Pen^2, D-Pen^5]Enkephalin at various concentrations (6–12 experimental points) was incubated with twice washed membranes of NG 108-15 or rat brain regions at 25°C for 9 hr (in NG 108-15) or 3 hr (in rat brain). All values were determined by nonlinear least-squares regression analysis (for one-site fit) based upon three independent experiments carried out in duplicate.

*An apparent dissociation constant (K_d) represents the geometric mean ±SEM.

†A maximal number of binding sites (B_{max}) represents the arithmetic mean ±SEM.

Table 2. Inhibition of [^{3}H][D-Pen^2, D-Pen^5]enkephalin or [^{3}H]naloxone binding by selected opioid compounds (enkephalins and narcotics) in the rat brain and NG 108-15 cell membrane preparations

	[^{3}H][D-Pen^2, D-Pen^5]Enkephalin		[^{3}H]Naloxone	
	NG 108-15 IC_{50},† nM	rat brain IC_{50},† nM	rat brain IC_{50}, nM	IC_{50} ratio*
[D-Pen^2, D-Pen^5]Enkephalin	0.82 ± 0.10	2.3 ± 0.3	2840‡	3463
[D-Pen^2, L-Pen^5]Enkephalin	1.58 ± 0.11	2.8 ± 0.4	3710‡	2348
[D-Pen^2, L-Cys^5]Enkephalin	0.52 ± 0.02	0.9 ± 0.3	178‡	342
[D-Thr^2, Leu^5, Thr^6]Enkephalin	3.55 ± 0.20	5.0 ± 0.2	36‡	10.1
[D-Ala^2, D-Leu^5]Enkephalin	0.51 ± 0.05	1.3 ± 0.1	16‡	31.4
Morphine	133.6 ± 2.1	86.8 ± 5.6	23‡	0.17
Tyr-D-Ala-Gly-MePhe-Gly-ol	306.8 ± 34.3	14.7 ± 0.8	1.3 ± 0.1	0.004
[MePhe^3, D-Pro^4]Morphiceptin	58,200	100,000	14	0.0002
Naloxone	73.3 ± 1.8	708 ± 116	1.0 ± 0.1	0.01

Various concentrations of each compound were incubated with [^{3}H][D-Pen^2, D-Pen^5]enkephalin (0.5 nM in NG 108-15 or 1.0 nM in whole rat brain less cerebellum) at 25°C for 9 hr (NG 108-15) or 3 hr (rat brain) in 50 mM Tris·HCl buffer (pH 7.4) containing 1 mg of bovine serum albumin and 100 μg of bacitracin per ml. The compounds also were tested against [^{3}H]naloxone binding in the whole rat brain preparation at 25°C for 1 hr in the same buffer. Values for IC_{50} were determined by nonlinear least-squares regression analysis based upon three or four independent experiments carried out in duplicate.

*IC_{50} of [^{3}H]naloxone in rat brain/IC_{50} of [^{3}H][D-Pen^2, D-Pen^5]enkephalin in NG 108-15.

†The concentrations at which half-maximal inhibition of ^{3}H-labeled ligands (IC_{50}) occur. Each value represents the geometric mean ±SEM.

‡Values are from refs. 6 and 7.

Saturation isotherms of [^{3}H][D-Pen^2, D-Pen^5]enkephalin binding also were performed for membrane preparations from discrete regions of the rat brain (Table 1). While similar K_d values (3.3–5.8 nM) were found in each brain region, there was an apparent regional distribution of the number of binding sites (cerebral cortex > hippocampus > pons-medulla > cerebellum).

Several opioid compounds were tested for their relative inhibitory potencies against specific [^{3}H][D-Pen^2, D-Pen^5]enkephalin binding in NG 108-15 and rat brain membrane preparations. For the NG 108-15 membrane preparation, all of the putative δ receptor-selective enkephalin analogues displayed high affinities, with IC_{50} values between 0.5 nM and 3.5 nM, and Hill slopes close to unity (Table 2). In contrast, [D-Ala^2, MePhe^4, Gly^5-ol]enkephalin, [MePhe^3, D-Pro^4]morphiceptin, morphine (all potent putative μ-receptor agonists), and naloxone (putative μ-receptor antagonist) were less effective inhibitors of [^{3}H][D-Pen^2, D-Pen^5]enkephalin binding than were the synthetic enkephalin analogues (14). A similar rank order of potency for these opioid compounds was observed in similar studies with rat brain membranes, except that [D-Ala^2, MePhe^4, Gly^5-ol]enkephalin had a relatively high affinity for [^{3}H][D-Pen^2, D-Pen^5]enkephalin-labeled sites (IC_{50} = 14.8 nM).

Although all of the enkephalin analogues tested inhibited [^{3}H][D-Pen^2, D-Pen^5]enkephalin binding in both NG 108-15 and rat brain homogenates, an interesting trend appeared when the ratios of IC_{50} values from [^{3}H]naloxone binding to rat brain (putative μ opioid receptor) and those for [^{3}H][D-Pen^2, D-Pen^5]enkephalin binding to NG 108-15 (δ opioid receptor) were used as an index of selectivity for δ versus μ opioid receptors (Table 2). Of the analogues examined, [D-Pen^2, D-Pen^5]enkephalin showed >3400-fold selectivity for the δ opioid receptor, whereas [D-Ala^2, D-Leu^5]enkephalin and [D-Thr^2, Leu^5, Thr^6]enkephalin showed only 31- and 10-fold selectivity, respectively, for the δ opioid receptor.

DISCUSSION

The present study provides the first *in vitro* characterization of [^{3}H][D-Pen^2, D-Pen^5]enkephalin binding in rat brain and NG 108-15 membranes. The kinetic data shows that association of [^{3}H][D-Pen^2, D-Pen^5]enkephalin to specific opioid sites in the two preparations proceeds at slow rates. At least 8–9 hr and 3 hr were necessary to allow the specific binding to reach steady state at 25°C in NG 108-15 and rat brain membranes, respectively. This slow association of binding also was observed with [^{3}H][D-Ala^2, D-Leu^5]enkephalin binding to NG 108-15 cell membranes under our conditions.

Parallel saturation studies with [^{3}H][D-Pen^2, D-Pen^5]enkephalin and [^{3}H][D-Ala^2, D-Leu^5]enkephalin in NG 108-15 membranes resulted in almost identical K_d and B_{max} values for the two ligands. This is not surprising when one considers that NG 108-15 membranes are thought to have only δ opioid receptors (3). Thus, both ligands bind to a single population of δ opioid receptors in this preparation. In support of this notion, Hill slopes obtained for both of these ligands were near 1.0.

All of the putative δ opiate receptor-selective enkephalin analogues tested displayed high affinities for [^{3}H][D-Pen^2, D-Pen^5]enkephalin-labeled sites in NG 108-15 membranes (IC_{50} = 0.51–3.55 nM) and rat brain membranes (IC_{50} = 0.94–5.1 nM). The ratio of IC_{50} values for the enkephalin analogue's ability to inhibit [^{3}H]naloxone in the rat brain (putative μ receptor) in the present and previous studies (6, 7) versus their ability to inhibit [^{3}H][D-Pen^2, D-Pen^5]enkephalin binding to NG 108-15 membranes in the present study provides a reasonable index of selectivity for δ versus μ opioid receptors. A striking improvement in selectivity of [D-Pen^2, D-Pen^5]enkephalin for the δ opioid receptor is apparent when the ratios for [D-Pen^2, D-Pen^5]enkephalin are compared with those for the prototypical δ opioid agonists [D-Ala^2, D-Leu^5]enkephalin and [D-Thr^2, Leu^5, Thr^6]enkephalin. The ratio for [D-Pen^2, D-Pen^5]enkephalin is 100- or 300-fold greater than those of [D-Ala^2, D-Leu^5]enkephalin or [D-Thr^2, Leu^5, Thr^6]enkephalin, respectively.

In conclusion, [^{3}H][D-Pen^2, D-Pen^5]enkephalin binds to δ opioid receptors with high affinity in two selected membrane preparations and is the most selective ligand for the δ opioid receptor of any enkephalin analogue developed thus far. [^{3}H][D-Pen^2, D-Pen^5]Enkephalin should be useful in the further characterization of the δ opioid receptor system, both *in vitro* and *in vivo*.

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