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

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ABSTRACT Specific binding properties of the tritium-labeled \(\delta\) 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 \(\mu\) receptor-selective ligands such as morphine, [D-Ala\(^2\), MePhe\(^4\), Gly\(^5\)-ol]enkephalin, [MePhe\(^4\), D-Pro\(^1\)]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 \(\delta\) opioid receptor.

It is well documented that the endogenous opioid pentapeptides, [Met\(^5\)enkephalin (H-Tyr-Gly-Phe-Met-OH) and [Leu\(^6\)enkephalin (H-Tyr-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 \(\delta\) opioid receptor was implemented by Mosberg et al. (4), who demonstrated that the cyclic conformationally constrained enkephalin analogues [D-Pen\(^2\), L-Cys\(^1\)]enkephalimide and [D-Pen\(^2\), D-Cys\(^1\)]enkephalimide displayed substantial \(\delta\) opioid receptor selectivity. Although the corresponding COOH-terminal analogues [D-Pen\(^2\), L-Cys\(^1\)]enkephalin and [D-Pen\(^2\), D-Cys\(^1\)]enkephalin were found to possess even more pronounced \(\delta\) 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; \(\beta\),\(\beta\)-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 \(\delta\) receptor selectivity. The selectivity of these analogues for the \(\delta\) 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 \(\delta\) opioid receptor than did the previously reported \(\delta\)-selective opioid agonists [D-Thr\(^5\), Leu\(^2\), Thr\(^3\)]enkephalin (9) or [D-Ser\(^2\), Leu\(^5\), Thr\(^3\)]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 \(\delta\) 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 \(\delta\) opioid receptors (3); thus, a comparison was made between \(\delta\) 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 \(\delta\) 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\(^1\)]enkephalin, and [D-Thr\(^5\), Leu\(^2\), Thr\(^3\)]enkephalin were synthesized by solid-phase methods and purified by partition and gel chromatography by previously described methods (4–6). [D-Ala\(^2\), L-Leu\(^5\)]Enkephalin and naloxone hydrochloride were purchased from Sigma and Du Pont, respectively.

Tyr-D-Ala\(^2\)-Gly-MePhe-Gly-ol (D-Ala\(^2\), MePhe\(^4\), Gly\(^5\)-ol)enkephalin and [MePhe\(^4\), D-Pro\(^1\)]morphiceptin were obtained from Peninsula Laboratories and K. J. Chang (Burrroughs 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\(^2\), L-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.

Abbreviation: Pen, penicillamine.

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homogenizer. The membranes were washed twice in the same buffer by centrifugation at 48,000 \times 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). \[^{[3H]}\text{D-Pen}^2, \text{D-Pen}^3\]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 \mu g/ml). \[^{[3H]}\text{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 \[^{[3H]}\text{Naloxone was used, incubations were carried out at 25°C for 1 hr. Nonspecific binding was defined as binding in the presence of 1 \mu M unlabeled \[^{\text{D-Pen}^2, \text{D-Pen}^3}\]enkephalin for \[^{[3H]}\text{D-Pen}^2, \text{D-Pen}^3\]enkephalin or 1 \mu M naltrexone in the case of \[^{[3H]}\text{Naloxone binding assays. In some studies \[^{[3H]}\text{D-Ala}^2, \text{D-Leu}^5\]enkephalin was investigated in parallel with \[^{[3H]}\text{D-Pen}^2, \text{D-Pen}^3\]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 non-linear 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 \[^{[3H]}\text{D-Pen}^2, \text{D-Pen}^3\]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 \[^{[3H]}\text{D-Pen}^2, \text{D-Pen}^3\]enkephalin in NG 108-15 membranes showed a linear relationship (correlation coefficient = 0.999) with protein concentrations up to 650 \mu g per assay. For routine assays, \(=90–220 \mu g of protein (5 \times 10^3 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-\mu l 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 \[^{[3H]}\text{D-Pen}^2, \text{D-Pen}^3\]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 \[^{[3H]}\text{D-Pen}^2, \text{D-Pen}^3\]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 \((\text{[L]}_t)\) was bound even at steady state, association of specific \[^{[3H]}\text{D-Pen}^2, \text{D-Pen}^3\]enkephalin binding to NG 108-15 membranes was plotted according to the pseudo-first-order equation (13):

\[
\ln \frac{[\text{LR}]_e}{[\text{LR}]_0 - [\text{LR}]} = k_+1 t + \ln \frac{[\text{LR}]_e}{[\text{LR}]_0 - [\text{LR}]},
\]

where \([\text{LR}]_0\) and \([\text{LR}]_e\) are the concentrations of ligand-receptor complex at steady state and at time \(t\) during incubation, respectively and \([\text{LR}]_t\) is the total concentration of binding sites. Linear regression (correlation coefficient = 0.999) of

\[
\ln \frac{[\text{LR}]_0}{[\text{LR}]_e - [\text{LR}]} = k_+1 t + \ln \frac{[\text{LR}]_0}{[\text{LR}]_e - [\text{LR}]},
\]

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

Dissociation of specifically bound \[^{[3H]}\text{D-Pen}^2, \text{D-Pen}^3\]enkephalin (0.6 nM) in NG 108-15 membranes was initiated
by the addition of unlabeled [d-Pen2, d-Pen7]enkephalin (final concentration of 1 µM) after 9 hr of incubation (steady state). Subsequent incubations in the presence of 1 µM [d-Pen2, d-Pen7]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-Pen2, d-Pen7]enkephalin) showed no deterioration. Dissociation was plotted (Fig. 2) according to the first-order equation (13):

$$\ln \left( \frac{LR}{LR_0} \right) = -k_{-1}t.$$

When a semi-logarithmic scale was used (Fig. 2 Inset), the plot linearly regressed (correlation coefficient = 0.997) and $k_{-1}$ was calculated from $0.5t_i = 3.45$ hr:

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

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 [3H][D-Pen7, D-Pen2]enkephalin and [3H][D-Ala2, D-Leu5]enkephalin binding in NG 108-15 membranes, respectively. The apparent dissociation constant ($K_d$) and the maximal number of binding sites ($B_{max}$) for [3H][D-Pen2, D-Pen7]enkephalin are almost identical to those for [3H][D-Ala2, D-Leu5]enkephalin. The $K_d$ (1.24 nM) for [3H][D-Pen2, D-Pen7]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 [3H][D-Pen7, D-Pen2]enkephalin and [3H][D-Ala2, D-Leu5]enkephalin binding were 1.10 (range, 1.06–1.16), and 0.96 (range, 0.93–1.35), respectively.

**Table 1.** [3H][D-Pen2, D-Pen7]Enkephalin binding to membranes from NG 108-15 cells and various rat brain regions

<table>
<thead>
<tr>
<th>Region</th>
<th>$K_d^*$ nM</th>
<th>$B_{max}^*$ fmol/mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>NG 108-15</td>
<td>1.24 ± 0.06</td>
<td>235.3 ± 13.1</td>
</tr>
<tr>
<td>Rat brain region</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>3.3 ± 0.2</td>
<td>117.0 ± 3.0</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>4.4 ± 0.8</td>
<td>64.6 ± 3.6</td>
</tr>
<tr>
<td>Pons-medulla</td>
<td>3.3 ± 0.4</td>
<td>39.0 ± 6.5</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>5.2 ± 1.7</td>
<td>32.2 ± 6.6</td>
</tr>
</tbody>
</table>

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

†A maximal number of binding sites ($B_{max}$) represents the arithmetic mean ± SEM.
Saturation isotherms of $[^3H]$d-Pen$_2$, d-Pen$_2$]-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 $[^3H]$d-Pen$_2$, d-Pen$_2$]-enkephalin binding in NG 108-15 and rat brain membrane preparations. For the NG 108-15 membrane preparation, all of the putative $\delta$ 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$_2$-ol]-enkephalin, [MePhe$_4$, d-Pro$_2$]Morpheicetin, morphine (all potent putative $\mu$-receptor agonists), and naltrexone (putative $\mu$-receptor antagonist) were less effective inhibitors of $[^3H]$d-Pen$_2$, d-Pen$_2$]-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$_2$-ol]-enkephalin had a relatively high affinity for $[^3H]$d-Pen$_2$, d-Pen$_2$]-enkephalin-labeled sites ($IC_{50} = 14.8$ nM).

Although all of the enkephalin analogues tested inhibited $[^3H]$d-Pen$_2$, d-Pen$_2$]-enkephalin binding in both NG 108-15 and rat brain homogenates, an interesting trend appeared when the ratios of $IC_{50}$ values from $[^3H]$naloxone binding to rat brain (putative $\mu$-opioid receptor) and those for $[^3H]$d-Pen$_2$, d-Pen$_2$]-enkephalin binding to NG 108-15 ($\delta$ opioid receptor) were used as an index of selectivity for $\delta$ versus $\mu$ opioid receptors (Table 2). Of the analogues examined, [d-Pen$_2$, d-Pen$_2$]-enkephalin showed $>3400$-fold selectivity for the $\delta$ opioid receptor, whereas [d-Ala$_2$, d-Leu$_2$]-enkephalin and [d-Thr$_3$, Leu$_3$, Thr$_3$]-enkephalin showed only $31$- and $10$-fold selectivity, respectively, for the $\delta$ opioid receptor.

**Table 2. Inhibition of $[^3H]$d-Pen$_2$, d-Pen$_2$]-enkephalin or $[^3H]$naloxone binding by selected opioid compounds (enkephalins and narcotics) in the rat brain and NG 108-15 cell membrane preparations**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>$[^3H]$d-Pen$_2$, d-Pen$_2$]-enkephalin</th>
<th>$[^3H]$Naloxone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NG 108-15 $IC_{50}$, nM</td>
<td>rat brain $IC_{50}$, nM</td>
</tr>
<tr>
<td>[d-Pen$_2$, d-Pen$_2$]-enkephalin</td>
<td>0.82 ± 0.10</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>[d-Pen$_2$, l-Pen$_2$]-enkephalin</td>
<td>1.58 ± 0.11</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>[d-Pen$_2$, l-Cys$_2$]-enkephalin</td>
<td>0.52 ± 0.02</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>[d-Thr$_2$, Leu$_2$, Thr$_3$]-enkephalin</td>
<td>3.55 ± 0.20</td>
<td>5.0 ± 0.2</td>
</tr>
<tr>
<td>[d-Ala$_2$, d-Leu$_2$]-enkephalin</td>
<td>0.51 ± 0.05</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Morphinose</td>
<td>133.6 ± 2.1</td>
<td>86.8 ± 5.6</td>
</tr>
<tr>
<td>Tyr-d-Ala-Gly-MePhe-Gly-ol</td>
<td>306.8 ± 34.3</td>
<td>14.7 ± 0.8</td>
</tr>
<tr>
<td>[MePhe$_4$, d-Pro$_2$]Morphiceptin</td>
<td>58,200</td>
<td>100,000</td>
</tr>
<tr>
<td>Naloxone</td>
<td>73.3 ± 1.8</td>
<td>708 ± 116</td>
</tr>
</tbody>
</table>

 Various concentrations of each compound were incubated with $[^3H]$d-Pen$_2$, d-Pen$_2$]-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 $\mu$g of bacitracin per ml. The compounds also were tested against $[^3H]$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 $[^3H]$naloxone in rat brain/IC$_{50}$ of $[^3H]$d-Pen$_2$, d-Pen$_2$]-enkephalin in NG 108-15.

*The concentrations at which half-maximal inhibition of $[^3H]$-labeled ligands (IC$_{50}$) occur. Each value represents the geometric mean ± SEM.

**Values are from refs. 6 and 7.**

All of the putative $\delta$ opiate receptor-selective enkephalin analogues displayed high affinities for $[^3H]$d-Pen$_2$, d-Pen$_2$]-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 $[^3H]$naloxone in the rat brain (putative $\mu$ receptor) in the present and previous studies (6, 7) versus their ability to inhibit $[^3H]$d-Pen$_2$, d-Pen$_2$]-enkephalin binding to NG 108-15 membranes in the present study provides a reasonable index of selectivity for $\delta$ versus $\mu$ opioid receptors. A striking improvement in selectivity for $[^3H]$d-Pen$_2$, d-Pen$_2$]-enkephalin for the $\delta$ opioid receptor is apparent when the ratios for [d-Pen$_2$, d-Pen$_2$]-enkephalin are compared with those for the prototypical $\delta$ opioid agonists [d-Ala$_2$, d-Leu$_2$]-enkephalin and [d-Thr$_3$, Leu$_3$, Thr$_3$]-enkephalin. The ratio for [d-Pen$_2$, d-Pen$_2$]-enkephalin is 100- to 300-fold greater than those of [d-Ala$_2$, d-Leu$_2$]-enkephalin or [d-Thr$_3$, Leu$_3$, Thr$_3$]-enkephalin, respectively.

In conclusion, $[^3H]$d-Pen$_2$, d-Pen$_2$]-enkephalin should be useful in the further characterization of the $\delta$ opioid receptor system, both in vitro and in vivo.

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