Specific protection of the binding sites of D-Ala\textsuperscript{2}-D-Leu\textsuperscript{5}-
enkephalin (\(\delta\)-receptors) and dihydromorphine (\(\mu\)-receptors)

By Linda E. Robson and H. W. Kosterlitz, F.R.S.
Unit for Research on Addictive Drugs, University of Aberdeen,
Marischal College, Aberdeen AB9 1AS, U.K.

(Received 3 May 1979)

Phenoxybenzamine causes a long-lasting inactivation of the opiate receptors of the \(\mu\) and \(\delta\)-type in homogenates of guinea-pig brain. This effect is selectively prevented when, before exposure to phenoxybenzamine, the homogenate is pre-incubated with ligands of high affinity for either of the two binding sites, i.e. dihydromorphine for the \(\mu\)-receptor and Tyr-D-Ala-Gly-Phe-D-Leu for the \(\delta\)-receptor. In contrast, Tyr-D-Ala-Gly-Phe-L-Leu amide, which has high affinities for both binding sites, protects both receptor sites.

Introduction

Differences in pharmacological profiles of opiates and opioid peptides in tests \textit{in vivo} and \textit{in vitro} have given rise to the question of whether they act on a homogeneous population of receptors (Martin \textit{et al.} 1976; Gilbert & Martin 1976; Lord \textit{et al.} 1976, 1977; Terenius 1977; Waterfield \textit{et al.} 1977; Chang \textit{et al.} 1978; Simantov \textit{et al.} 1978; Kosterlitz \textit{et al.} 1979). In particular, comparison of the results of binding studies in brain with those of bioassays in preparations innervated by cholinergic or adrenergic nerves has suggested that the opiate receptor populations in both the central and peripheral nervous systems are heterogeneous (Lord \textit{et al.} 1976, 1977). Non-peptide opiate agonists and antagonists are more potent inhibitors of the binding of labelled opiates than of labelled enkephalins, whereas the reverse is true of enkephalins and their analogues; it is of particular interest that the long-chain peptide \(\beta\)-endorphin is equally potent in inhibiting both types of labelled ligands.

These conclusions are based on the observation that the rank order of potency is different in different assay systems. Evidence of a more direct nature would be obtained if it could be shown that the appropriate ligands specifically protect the binding sites against inactivation by alkylating agents. Thus, it would be expected that, for the protection of the opiate binding sites, opiates of the alkaloid type would protect the opiate binding sites in lower concentrations than the enkephalins and that, conversely, the enkephalin binding sites would be more readily protected by enkephalins than by opiates. Phenoxybenzamine has been chosen as an alkylating agent since it has been shown (Cicero \textit{et al.} 1974, 1975; Spiehler \textit{et al.} 1978)
that it inhibits the specific binding of [³H]naloxone irreversibly, an effect which is prevented by simultaneous incubation with cold levallorphan (N-allyl-3-hydroxymorphanan). In the present investigation [³H]Tyr-D-Ala-Gly-Phe-D-Leu was used as a primary ligand for testing the enkephalin or δ-binding site and [³H]dihydromorphine for testing the morphine or μ-binding site (Lord et al. 1977; Kosterlitz et al. 1979). The same compounds served as protecting cold ligands; Tyr-D-Ala-Gly-Phe-L-Leu amide was used in addition because it has high affinities for both the μ- and δ-receptor binding sites (Gillan et al. 1979).

**Materials and methods**

(a) **Experimental procedure**

The method used for preparing homogenates of guinea-pig brain was a modification of that described by Pert & Snyder (1973) and Lord et al. (1976).

The principles of the experimental procedure are shown in scheme 1. The reasons for the various steps are as follows. In step 1 cell detritus and nuclei were removed because binding to these constituents reduced reproducibility. The supernatant obtained after centrifugation at 300 g was centrifuged at 49000 g for 10 min and the resulting pellets resuspended in Tris buffer to give a concentration of 45 mg wet mass of original brain per millilitre. The specific binding of tritiated ligands did not differ between aliquots of this improved homogenate.

In step 2, concentrations of cold ligands were used to give between 20 and 80% protection against the effect of a concentration of phenoxybenzamine which resulted in 70–80% inhibition of binding. The homogenate was pre-incubated at 37 °C for 10 min with the protecting cold ligand and then incubated for 15 min with a mixture of phenoxybenzamine and cold ligand. Although D-Ala²-D-Leu⁵-enkephalin is much more resistant to the action of peptidases than the natural enkephalin, an unknown loss was to be expected during incubation. This fact may result in too small a protecting effect of the enkephalin analogue but does not vitiate the validity of the design which is based on the crossover principle.

Steps 3 and 4 have the purpose of removing all but traces of the cold protecting ligands. When, in one experiment, small amounts of tritiated ligand were added to the cold ligands, it was found that steps 3 and 4 removed 99% of dihydromorphine and D-Ala²-D-Leu⁵-enkephalin and 98% of D-Ala²-L-Leu⁵-enkephalin amide. When in step 2 dihydromorphine (384 nM), D-Ala²-D-Leu⁵-enkephalin (384 nM) or D-Ala²-L-Leu⁵-enkephalin amide (48 nM) were added without phenoxybenzamine, the binding of [³H]dihydromorphine or of [³H]D-Ala²-D-Leu⁵-enkephalin after steps 3 and 4 was the same as in the homogenate to which no peptide had been added (two experiments).

In step 5 either [³H]D-Ala²-D-Leu⁵-enkephalin (1.4 nM) (scheme 1) or [³H]-dihydromorphine (0.6 nM) was used to determine the percentage of binding sites that had been protected by various concentrations of the two cold ligands against
the inactivating effect of phenoxybenzamine. The protection effected by a particular concentration of cold ligand was calculated as the difference in specific binding between homogenate treated with phenoxybenzamine only and phenoxybenzamine plus protecting ligand. The degree of protection was expressed as a percentage of that amount of specific binding that had been inhibited by phenoxybenzamine.

**Scheme 1. Experimental procedure to show the specific protection by cold ligands of binding of [³H]D-Ala²-D-Leu-ENKEPHALIN FROM THE INHIBITORY EFFECT OF PHENOXYBENZAMINE**

**step 1** prepare brain homogenate from which the P₁ fraction has been removed by centrifugation at 300 g for 15 min (45 mg brain wet mass per millilitre Tris buffer (50 nM, pH 7.4 at 37 °C))

**step 2** incubate at 37 °C

(a) from 0 to 10 min with unlabelled di-D-Ala²-D-Leu⁵-enkephalin (1.5–24 nM)† or hydromorphine (24–384 nM)†

(b) from 10 to 25 min with cold ligands and with phenoxybenzamine (2.4 μM)

**step 3** centrifuge at 49000 g for 10 min, resuspend pellet in Tris buffer and incubate at 37 °C for 15 min

**step 4** repeat step 3

**step 5** centrifuge at 49000 g for 10 min, resuspend pellet in Tris buffer (pH 7.4 at 25 °C), determine binding of [³H]D-Ala²-D-Leu⁵-enkephalin (1.4 nM) at 25 °C for 40 min.

† When [³H]dihydromorphine (0.6 nM) was the tritiated ligand, the concentrations of unlabelled D-Ala²-D-Leu⁵-enkephalin were 24–384 nM and those of unlabelled dihydromorphine 3–48 nM.

The specific binding of either [³H]dihydromorphine (0.6 nM) or [³H]D-Ala²-D-Leu⁵-enkephalin (1.4 nM) was determined in triplicate for each concentration of cold ligand. After the homogenate (1.9 ml) had been made up to 2.0 ml with Tris–HCl buffer, pH 7.4 at 25 °C, and tritiated ligand, it was incubated for 40 min at 25 °C and filtered. The GF/B glass fibre filters were incubated with 1 ml Soluene at 60 °C, and after neutralization and addition of 10 ml Unisolve, the radioactivity was determined in a scintillation counter at an efficiency of 35–40%. Specific binding was calculated from the difference of the counts in the absence and in the presence of an excess of inhibiting drug, 250 nM Mr 2266 or 200 nM dihydromorphine for binding of [³H]dihydromorphine and 2.5 μM Mr 2266 or 100 nM D-Ala²-D-Leu⁵-enkephalin for binding of [³H]D-Ala²-D-Leu⁵-enkephalin. Specific binding of [³H]D-Ala²-D-Leu⁵-enkephalin varied between 62 and 75% and of [³H]dihydromorphine between 53 and 77%.

**b) Peptides and drugs**

These were: D-Ala²-D-Leu⁵-enkephalin (Dr S. Wilkinson, Wellcome Research Laboratories); D-Ala²-Leu-enkephalin amide (Dr J. S. Morley, Imperial Chemical Industries); dihydromorphine HCl (Dr N. B. Eddy); phenoxybenzamine HCl
(Smith, Kline and French); (—)-2-(3-furylmethyl)-5,9-diethyl-2'-hydroxy-6,7-benzomorphan (Mr 2266) as the free base (Dr H. Merz, Boehringer-Ingelheim). Stock solutions (usually 1 mg/ml) were made up in distilled water and stored at —20 °C. For drugs used as the free base, the calculated amount of HCl was added. Peptides were stored in plastic vials.

(c) Tritiated ligands

Both ligands were obtained from the Radiochemical Centre, Amersham. The specific activity of [3H]dihydromorphine varied between 74 and 81 Ci/mmol and that of [3H]D-Ala²-D-Leu⁵-enkephalin between 44 and 48 Ci/mmol.

**Results**

(a) Inhibition of binding by phenoxybenzamine

The inhibitory effect of phenoxybenzamine was dose-related, leading to complete loss of binding at high concentrations. When, in a series of paired experiments, the effects of phenoxybenzamine on the two ligands was tested in the same homogenate, it was found that the binding of [3H]dihydromorphine or [3H]D-Ala²-D-Leu⁵-enkephalin was inhibited equally well, the i.c.₅₀ values being 0.84 ± 0.11 μM and 1.28 ± 0.06 μM (n = 3), respectively (figure 1). An inhibition of about 70% binding was considered desirable for the protection experiments and was obtained with 2.4 μM phenoxybenzamine.

![Figure 1. Inhibition by phenoxybenzamine of binding of [3H]dihydromorphine (0.6 nM) and [3H]D-Ala²-D-Leu⁵-enkephalin (1.4 nM) in homogenates of guinea-pig brain. ○, [3H]-dihydromorphine; ▲, [3H]D-Ala²-D-Leu⁵-enkephalin. The regressions of logarithm of concentration on percentage inhibition were calculated from three experiments, each point representing two or three observations; in each experiment the binding of both tritiated ligands was determined in the same homogenate.](http://rsbp.royalsocietypublishing.org/Downloaded from 20170131)
Specific protection by cold ligands against the inhibitory effect of phenoxybenzamine

The protecting effects of the cold ligands D-Ala²-D-Leu⁵-enkephalin and dihydromorphine are shown in figure 2. When [³H]D-Ala²-D-Leu⁵-enkephalin was the tritiated ligand, much less D-Ala²-D-Leu⁵-enkephalin than dihydromorphine was required for the same protecting effect (figure 2a). The reverse holds when [³H]dihydromorphine was used as the tritiated ligand (figure 2b). Since D-Ala²-L-Leu⁵-enkephalin amide has been shown (Gillan et al. 1979) to combine with a considerably greater number of binding sites than either dihydromorphine or D-Ala²-D-Leu⁵-enkephalin, its protecting power was expected to be less selective.

Figure 2. Protection of the binding in homogenates of guinea-pig brain of (a) [³H]D-Ala²-D-Leu⁵-enkephalin (1.4 nM) and (b) [³H]dihydromorphine (0.6 nM) from the inhibitory effect of phenoxybenzamine (2.4 μM). The protecting effects of unlabelled D-Ala²-D-Leu⁵-enkephalin and dihydromorphine were always tested in the same homogenate. Each point is the mean of from three to five observations. The regressions of logarithm of concentration of cold ligand on percentage protection were calculated from five experiments. •, Dihydromorphine; ○, D-Ala²-D-Leu⁵-enkephalin. The mean concentrations of the cold ligands (i.e., 50 ± s.e.m.) required to protect 50% of the binding are indicated by ■ and □. The inhibition of binding by phenoxybenzamine was 77.7 ± 2.1% for [³H]D-Ala²-D-Leu⁵-enkephalin and 78.2 ± 2.7% for [³H]dihydromorphine.
When dihydromorphine was the tritiated ligand, the amide was as potent a protecting agent as cold dihydromorphine; conversely, when $[^3\text{H}]\text{d-Ala}^2\text{-d-Leu}^5$-enkephalin was used, the amide was equipotent with cold $\text{d-Ala}^2\text{-d-Leu}^5$-enkephalin in protecting the binding sites.

![Figure 3](http://rsbp.royalsocietypublishing.org/Downloaded from)

**Figure 3.** Protection of the binding in homogenates of guinea-pig brain of (a) $[^3\text{H}]\text{d-Ala}^2\text{-d-Leu}^5$-enkephalin (1.4 nM) and (b) $[^3\text{H}]$dihydromorphine from the inhibitory effect of phenoxybenzamine (2.4 μM). The protecting effects of the cold ligands were always tested in the same homogenate. Each point is the mean of two or three observations. The regressions of logarithm of concentration of cold ligand on percentage protection were calculated from three experiments. •, Dihydromorphine; ○, $\text{d-Ala}^2\text{-d-Leu}^5$-enkephalin; △, $\text{d-Ala}^2\text{-l-Leu}^5$-enkephalin amide. The mean concentrations (i.e., ± s.e.m.) of the three cold ligands required to protect 50% of the binding from phenoxybenzamine are indicated by ■, □, ▲. The inhibition of binding by phenoxybenzamine was 88.6 ± 3.6% for $[^3\text{H}]\text{d-Ala}^2\text{-d-Leu}^5$-enkephalin and 85.4 ± 4.9% for $[^3\text{H}]$dihydromorphine.

These data were subjected to an analysis by Hill plots (table 1). The linear correlation coefficients were satisfactory and the Hill coefficients indicated a slope of unity. The concentrations giving 50% protection showed highly significant ligand selectivity as far as dihydromorphine or $\text{d-Ala}^2\text{-d-Leu}^5$-enkephalin was concerned. On the other hand, $\text{d-Ala}^2\text{-l-Leu}^5$-enkephalin amide was equipotent with the respective cold ligand in protecting the binding sites of either $[^3\text{H}]$dihydromorphine or $[^3\text{H}]\text{d-Ala}^2\text{-d-Leu}^5$-enkephalin.
TABLE 1. SPECIFIC PROTECTION FROM THE INACTIVATING EFFECT OF PHENOXYBENZAMINE (2.4 μM) ON OPIATE RECEPTORS IN HOMOGENATES OF GUINEA-PIG BRAIN

<table>
<thead>
<tr>
<th>protecting cold ligands</th>
<th>correlation coefficient</th>
<th>Hill slope</th>
<th>iC50/μM</th>
<th>difference/μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Protection of [3H]dihydromorphine binding (0.6 nM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dihydromorphine</td>
<td>0.954 ± 0.017</td>
<td>1.08 ± 0.17</td>
<td>13.1 ± 3.2</td>
<td>+ 60.6 ± 12.2* (5)</td>
</tr>
<tr>
<td>D-Ala2-D-Leu5-enkephalin</td>
<td>0.981 ± 0.004</td>
<td>1.25 ± 0.16</td>
<td>73.7 ± 12.8</td>
<td></td>
</tr>
<tr>
<td>dihydromorphine</td>
<td>0.955 ± 0.003</td>
<td>1.17 ± 0.19</td>
<td>16.5 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>D-Ala2-L-Leu5-enkephalin amide</td>
<td>0.989 ± 0.004</td>
<td>0.95 ± 0.05</td>
<td>14.4 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>(b) Protection of [3H]D-Ala2-D-Leu5-enkephalin binding (1.4 nM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Ala2-D-Leu5-enkephalin</td>
<td>0.963 ± 0.011</td>
<td>1.13 ± 0.16</td>
<td>4.64 ± 0.42</td>
<td></td>
</tr>
<tr>
<td>dihydromorphine</td>
<td>0.960 ± 0.013</td>
<td>1.20 ± 0.29</td>
<td>90.5 ± 8.1</td>
<td>+ 85.9 ± 8.22** (5)</td>
</tr>
<tr>
<td>D-Ala2-D-Leu5-enkephalin</td>
<td>0.943 ± 0.016</td>
<td>1.34 ± 0.36</td>
<td>5.40 ± 1.43</td>
<td></td>
</tr>
<tr>
<td>D-Ala2-L-Leu5-enkephalin amide</td>
<td>0.945 ± 0.028</td>
<td>1.25 ± 0.09</td>
<td>5.50 ± 1.00</td>
<td>+ 0.10 ± 2.42 (3)</td>
</tr>
</tbody>
</table>

The values are the means ± s.e.m.; the numbers of observations are given in parentheses. The data were calculated from the regression of log10 (concentration of cold ligand) on log10 (protected count)/(unprotected count). Significance levels: *, p < 0.005; **, p < 0.0005.

DISCUSSION

The experiments described in this paper present the first direct evidence for the existence in homogenates of guinea-pig whole brain of two binding sites with different affinities to opiate alkaloids and opioid peptides. Incubation with phenoxybenzamine in a concentration of 0.8–1.3 μM reduced the binding of either [3H]dihydromorphine or [3H]D-Ala2-D-Leu5-enkephalin by 50%, a value similar to that previously found for the inhibition of binding of [3H]naloxone (1.2–1.4 μM) (Cicero et al. 1975; Spiehler et al. 1978). This effect was not reversed when the homogenate was incubated at 37 °C for two periods of 15 min although this treatment removed all but 1–2% of reversibly bound dihydromorphine, D-Ala2-D-Leu5-enkephalin or D-Ala2-L-Leu5-enkephalin amide. These findings are in agreement with the observations of Spiehler et al. (1978) that the interaction of phenoxybenzamine with the opiate receptors in homogenates of mouse brain is irreversible.

This irreversible inactivation of the binding sites can be prevented selectively when the homogenate is pretreated with the appropriate cold ligand before the addition of phenoxybenzamine. When [3H]dihydromorphine binding was tested, cold dihydromorphine was six times more potent in protecting the binding site than D-Ala2-D-Leu5-enkephalin. In contrast, for the protection of D-Ala2-D-Leu5-binding it was necessary to use 20 times more dihydromorphine than D-Ala2-D-Leu5-enkephalin. While these findings indicate the presence of two binding sites, there is cross reactivity between the receptor sites. These observations confirm...
the view (Kosterlitz et al. 1979) that dihydromorphine binds preferentially to its own binding sites (μ-receptor) but in higher concentration also to the binding site represented by D-Ala²-D-Leu⁵-enkephalin (δ-receptor). The converse holds true for D-Ala²-D-Leu⁵-enkephalin which preferentially binds to the δ-receptor and in higher concentration to the μ-receptor. This concept is supported by the observation that cold n-Ala²-L-Leu⁵-enkephalin amide, which binds readily to both binding sites (Gillan et al. 1979), protects the δ-receptor as well as cold D-Ala²-D-Leu⁵-enkephalin and the μ-receptor as effectively as cold dihydromorphine.

Finally, it will be of importance to search for even more specific ligands than those used in the present investigation because they will yield more information of the extent of cross reactivity and also will facilitate the identification of the two receptor sites in different regions of the brain.

The work was supported by the Medical Research Council, the U.S. National Institute on Drug Abuse (DA 00662) and the U.S. Committee on Problems of Drug Dependence. Acknowledgement is made of generous gifts of the compounds mentioned in Methods.

References


