Effects of Double-enkephalin (Biphalin), an Enkephalin Analogue, on Respiration and the Cough Reflex in Rats

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The pharmacological actions of double-enkephalin (biphalin; (HCl-Try-d-Ala-Gly-Phe-NH₂)₂), an analogue of enkephalin, on nociception, respiration and the cough reflex were compared with those of morphine in anesthetized rats. Double-enkephalin (D-Enk), injected i.p., produced significant analgesia at doses of 10 and 20 mg/kg in a hot-plate test. The analgesic effect of D-Enk was antagonized by pretreatment with naloxone (5 mg/kg, i.p.). D-Enk and morphine (M) produced a dose-dependent decrease in the frequency of respiration (RF) and in the tidal volume (Vₐ). However, the effects of D-Enk on RF and Vₐ were significantly weaker than those of M. The 50% antitussive dose (AtD50) of D-Enk and M were 0.63 and 0.48 mg/kg, i.p., respectively. The antitussive effect of D-Enk was antagonized by pretreatment with naloxone (0.4 mg/kg, i.p.). These results suggest that D-Enk exerted an antitussive effect similar to that of morphine, and that the involvement of opiate receptors is associated with the antitussive effect of D-Enk.

Keywords — enkephalin analogue; double-enkephalin; cough reflex; respiration; morphine

Introduction

Since the discovery of the endogenous, opiate-like peptides, methionine- and leucine-enkephalin, numerous analogues of these substances have been synthesized and various structure-activity relationships established. 1-10 These studies have led to the following conclusions. 1) The amino-terminal tyrosine residue is essential for the activity of the opiate-like, peptide analogues of enkephalin. 2) The replacement of the glycine residue in position 2 by a D-amino acid residue enhances both resistance to enzymatic degradation and binding to receptors, and, in consequence, increases the biological activity of the analogues.

Lipkowski et al. 6) synthesized an analogue of enkephalin, called double-enkephalin (biphalin; D-Enk), in which the C-terminal methionine or leucine residue is replaced by a second active fragment of the enkephalin analogue. The two fragments of the D-Ala²-enkephalin analogue are connected by a diamine bridge to give the following formula:

\[
\text{Try-D-Ala-Gly-Phe-NH} \quad \mid \quad \text{Try-D-Ala-Gly-Phe-NH}
\]

D-Enk produced a potent, naloxone-reversible analgesia when administered intraperitoneally to mice.

The effects of D-Enk on respiratory function and the cough reflex have not yet been examined even though they are of particular interest since the depression of respiration and the antitussive effect are actions characteristic of opiates. 11) Thus, in the present study, we have compared the respiratory-depressant and antitussive properties of D-Enk with those of morphine in rats.

Methods and Materials

Animals — Male Sprague-Dawley rats weighing 230-380 g were used throughout. The rats were housed under alternating 12-h light-dark cycles and given normal food and water. The animals were used only once.

Antinociception Study — Antinociception was evaluated in the rat using a hot plate. The delay in the perception of pain on the hot plate was determined by placing the animal on a heated copper plate (25 cm × 20 cm), maintained at 55 °C, waiting until the rat licked any of its paws. A cut-off time of 30 s was used in
this test to prevent burning of the paws, and the delay or latency in such cases was taken as 30 s. Measurements of latency were made 30 min before administration of drugs, and 15, 30 and 60 min after the administration of the drugs. All drugs were administered intraperitoneally (i.p.). Naloxone was administered i.p. 5 min before administration of morphine or double-enkephalin.

Measurement of Respiration — The rats were anesthetized with \( \alpha \)-chloralose (70 mg/kg, i.p.). An incision was made through the strap muscles of the neck to expose the trachea, and a tracheal cannula was inserted into a caudal site of the transected trachea. The animals were able to breathe spontaneously through this cannula. Respiration was recorded from the tracheal cannula using a pneumotachograph (Nihon Kohden, TP-602T) connected to a polygraph (Nihon Kohden, RM-6100), which permitted quantification of tidal volume and frequency of respiration. Drugs were administered intravenously through a cannula inserted into the femoral vein. Dose-response curves were generated by cumulative intravenous administration of morphine or double-enkephalin.

Induction of the Cough Reflex — Animals were fixed in a dorsal position under \( \alpha \)-chloralose anesthesia (70 mg/kg, i.p.). The cough reflex was induced by electrical stimulation, by the puncture electrode-induced cough method.\(^{12}\) A puncture electrode was made of stainless-steel wire (0.2 mm diameter, 10 cm length) and coated with epoxy for insulation. The electrode was inserted into the trachea through a guiding cannula (injection needle 23G, Terumo). The guiding cannula was pulled out as soon as the electrode had been inserted into the trachea. A stainless-steel injection needle, placed arbitrarily into the muscle behind the ear, was used as the indifferent electrode with reference to the electrode inserted into the trachea. The electrical stimulation used for inducing the cough reflex consisted of a square-wave pulse with a frequency of 40 Hz; the duration of the pulse was 1 ms; the voltage was 2—4 V; and the duration of application was 10 s. The stimulus intensity for each animal was set by increasing the voltage. The thoracic movements of the rat were measured with a force-displacement transducer (Nihon Kohden, TB-611T) and used as an indicator of the cough reflex. Recordings were made on a polygraph (Nihon Kohden, RM-6100). The electrical stimuli used for inducing the cough reflex were given at 5, 10, 15, 30, 45 and 60 min after administration of drugs. When no cough reflex occurred in response to even one stimulus, the drug was regarded as effective. When the cough reflex occurred in response to all stimuli, the drug was regarded as ineffective. Only one dose of drug was given to each animal. A minimum of 6 animals was used for each dose of each drug. All drugs were administered intraperitoneally (i.p.). Naloxone was administered i.p. 5 min before administration of morphine or double-enkephalin.

Chemicals — The following drugs were used in this study: double-enkephalin (biphalin) (provided by Dr. A. W. Lipkowski, Warsaw University, Warsaw, Poland), morphine hydrochloride (Sankyo, Tokyo) and naloxone hydrochloride (Endo Lab., Garden City, NY). All drugs were dissolved in saline immediately before use. Doses of all drugs are expressed as the base.

Analysis of Data — The significance of changes in latencies during the hot-plate test and in respiratory parameters before and after administration of drugs were determined by the Student’s t-test. The 50% antitussive doses of morphine and double-enkephalin were determined by a log-probit analysis of the dose-response data.

Results

1. Effects on Pain Thresholds

The mean control latency in the hot-plate test was 7.07 ± 0.62 s. Pain thresholds after administration of morphine and double-enkephalin (D-Enk) are presented in Fig. 1. D-Enk produced statistically significant analgesia 15, 30, and 60 min after administration at doses of 10 and 20 mg/kg. The higher dose (20 mg/kg) of D-Enk increased the latency of the response 60 min after administration by 177.4% of the pre-injection control value, and by 92.2% of the effect of double the equimolar dose of morphine.
Double-enkephalin and Cough Reflex

![Graph showing pain thresholds of rats treated with D-Enk (10 mg/kg and 20 mg/kg) or Morphine (10 mg/kg).](image)

The numbers in each column indicate the dose in mg/kg of body weight. Naloxone was injected 5 min before the administration of D-Enk. Each column represents the mean with S.E. of results from five experiments. Significant differences from control values obtained with saline are indicated by a) $p < 0.05$ and b) $p < 0.01$. Significant differences from D-Enk (20 mg/kg) alone are depicted by c) $p < 0.05$ and d) $p < 0.01$. All drugs were injected intraperitoneally.

(10 mg/kg). The analgesic effect of D-Enk was antagonized by pretreatment with naloxone (5 mg/kg, i.p.).

2. Effects of Cumulative Doses of D-Enk and Morphine on Tidal Volume and Frequency of Respiration

D-Enk and morphine produced a decrease in tidal volume ($V_t$) and frequency of respiration (RF). These effects reached their maximum 15 min after the administration of either of the two drugs. Thus, an interval of 15 min after administration was chosen for experiments designed to quantitate the effects of D-Enk and morphine (M) on respiration.

The effects of cumulative doses of D-Enk and M on RF are shown in Fig. 2. D-Enk and morphine in doses from 0.1 to 30 mg/kg decreased the RF in a dose-dependent manner. As shown in Fig. 3, intravenous administration of D-Enk and M resulted in a dose-dependent decrease in $V_t$. The decreases in RF and $V_t$ caused by D-Enk, however, were markedly smaller than those caused by morphine.

3. Effects of D-Enk and Morphine on the Cough Reflex

Dose-response data for the effects of intraperitoneally administered D-Enk and M on the cough reflex are shown in Fig. 4. The 50% ant
Table I. The AtD_{50} Values of Double-enkephalin (D-Enk) and Morphine in the Absence or Presence of Naloxone

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>AtD_{50} (mg/kg, i.p.)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>D-Enk</td>
</tr>
<tr>
<td>None</td>
<td>0.63</td>
</tr>
<tr>
<td>Naloxone (0.4 mg/kg, i.p.)</td>
<td>1.92</td>
</tr>
</tbody>
</table>

AtD_{50}, 50% antitussive dose. AtD_{50} values were determined by probit analysis of dose-response data. Naloxone was administered 5 min before D-Enk and morphine.

tussive doses (AtD_{50}) of D-Enk and M (dose required to depress the cough reflex in 50% of rats tested) were 0.63 and 0.48 mg/kg, respectively (Table 1). Pretreatment with naloxone shifted the dose-response curves in parallel and to the right of the dose-response curves for D-Enk and M, respectively (Fig. 4). The AtD_{50} of D-Enk and M for rats pretreated with naloxone (0.4 mg/kg, i.p.) was 3-fold higher in each case than the corresponding AtD_{50} for rats that were not pretreated with naloxone.

Discussion

The present investigation was undertaken to study the cough-depressant effect of D-Enk, a synthetic analogue of enkephalin. D-Enk administered systemically exerted potent and naloxone-reversible antitussive and antinociceptive effects. D-Enk and morphine were active over the same range of antitussive doses (0.3–1.0 mg/kg), and their potencies were not significantly different (AtD_{50} for D-Enk and morphine are 0.63 and 0.48 mg/kg, i.p., respectively.) It is noteworthy that the antitussive and antinociceptive potencies of D-Enk were about two-fold higher than the analogues potencies of equimolar doses of morphine. Several possibilities may account for the relatively higher biological activity of D-Enk and should be considered. First, one of the most promising ways of developing new opiate analogues is via the synthesis of compounds containing two active elements in one molecule. The two active elements in such compounds may link vicinal or adjacent
receptors. Such compounds appear to have considerably higher biological activities because of their ability to bind with more than one receptor per molecule. Furthermore, the amino-terminal tyrosine is important for the activity of analogues of enkephalin.\textsuperscript{4} D-Enk, containing two tyrosine residues per molecule has two active sites for interaction with the opiate receptor(s). In addition, one active element would still remain even if one of the active elements of D-Enk was inactivated by enzymatic degradation. It is possible that the unique properties of D-Enk may account for its higher antitussive and antinociceptive activities. Second, some studies indicate that the interactions of opiate agonists at mu, delta, or kappa receptors can mediate analgesia.\textsuperscript{14,15} On the other hand, the fact that the kappa agonist cyclazocine and ketocyclazocine can inhibit the cough reflex\textsuperscript{16} suggests that the cough-depressant effect can also be mediated by multiple interactions with opiate receptors. Furthermore, D-Enk shows significant affinity for all three subtypes of opiate receptors (mu, delta, and kappa) in brain membranes, whereas morphine exhibits a high degree of selectivity for the mu receptor.\textsuperscript{13} It seems likely, therefore, that its relatively high antitussive and antinociceptive activities may be due to the high affinity and low selectivity of D-Enk for opiate receptors.

D-Enk produced a dose-dependent reduction of both $V_t$ and RF. The ED$_{50}$'s of D-Enk for the reduction of $V_t$ (13.6 mg/kg) and RF (10.2 mg/kg) are not markedly different from each other. Morphine also reduced both the $V_t$ and the RF in a dose-dependent manner. However, morphine was less effective in reducing RF than in reducing $V_t$. The ED$_{50}$ of morphine for the reduction of RF (5.5 mg/kg) is 3 times higher than the ED$_{50}$ for the reduction of $V_t$ (1.6 mg/kg). In studies of the specific involvement of opiate receptors in the opiate-induced depression of respiration, several investigators have concluded that mu receptors are involved in the reduction of $V_t$, while delta receptors are more specifically involved in the decrease in RF.\textsuperscript{17,18} It is well-known that morphine has been classified as a selective agonist of mu receptors. In addition, low doses of morphine that cause a slight reduction in $V_t$ do not change the RF.\textsuperscript{19} However, D-Enk exhibits a similar affinity for mu and delta receptors.\textsuperscript{13} Therefore, the differential effects induced by D-Enk and morphine upon $V_t$ and RF could be the result of the differential selectivities for opiate receptors (mu and delta).

Systemically administered D-Enk exhibits a much greater antitussive effect than morphine when these drugs are compared on a molar basis. In contrast, D-Enk has a significantly lower respiratory-depressant effect than morphine. Thus, D-Enk exhibits a marked antitussive effect with a less significant effect on respiration. From the present study alone, we cannot clarify the mechanism that explains this unique property on D-Enk. This property, however, makes D-Enk an effective and useful antitussive drug.

On the basis of our results it is not possible to differentiate between central and peripheral factors contributing to the antitussive effect after systemic administration of D-Enk. Furthermore, it is unclear at present, whether D-Enk can easily penetrate the blood-brain barrier. Most narcotic and non-narcotic antitussive drugs, however, have their site of action in the central nervous system (CNS). Thus, one can speculate that D-Enk exhibits its antitussive effect through the CNS.

In summary, we have demonstrated that D-Enk produces a much greater antitussive effect than morphine when these drugs are compared on a molar basis, and the antitussive effects of D-Enk appear to be mediated directly \textit{via} opiate receptors.

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\textbf{References}


