Pharmacology Biochemistry & Behavior, Vol. 12, pp. 645–649 Printed in the U.S.A.

Comparative Effects of Synthetic Enkephalinamides and Morphine on Abstinence Responses in Morphine-Dependent Mice

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Received 25 August 1979

BHARGAVA, H. N. *Comparative effects of synthetic enkephalinamides and morphine on abstinence responses in morphine dependent mice.* PHARMAC. BIOCHEM. BEHAV. 12(5) 645–649, 1980.—The effects of intracerebroventricular (ICV) administration of two synthetic enkephalinamides, D-Ala^2-Met^5-enkephalinamide and D-Met^2-Pro^5-enkephalinamide, were compared with those of morphine on abstinence responses in morphine dependent mice. Mice were rendered dependent by morphine pellet implantation for three days. When administered six hours after pellet removal, both enkephalinamides and morphine reversed the naloxone-induced abstinence jumping response. On a molar basis, D-Met^2-Pro^5-enkephalinamide was the most potent compound and morphine was the least potent. Thus, substitution of D-Methionine in the two-position and prolineamide in five-position of the naturally occurring enkephalins, methionine- and leucine-enkephalin resulted in potent compound. The enkephalinamides and morphine also prevented hypothermia when injected immediately after or six hours after morphine withdrawal (pellet removal).

D-Met^2-Pro^5-enkephalinamide D-Ala^2-Met^5-enkephalinamide Morphine Naloxone Dependence Abstinence syndrome Hypothermia

IN recent years considerable *in vivo* and *in vitro* evidence indicates that peptides like leucine-enkephalin (H-Tyr-Gly-Gly-Phe-Leu-OH) and various fragments of β-lipotropin (91 amino acids) can act as endogenous ligands for the opiate receptors in various mammalian tissues, particularly the brain [1, 4, 10]. The amino acid sequences of methionine-enkephalin, α-endorphin, and β-endorphin correspond to residues 61–65, 61–76, and 61–91 of β-lipotropin, respectively. However, the naturally occurring enkephalins and endorphins do not cross the blood brain barrier and exhibit pharmacological profile similar to that of morphine only when injected directly in the brain [8]. Furthermore, these peptides possess short duration of action because of their rapid degradation by the proteolytic enzymes of the tissue. Although methionine-enkephalin and β-endorphin produce withdrawal signs like teeth chattering and escape responses when perfused continuously into the periaqueductal gray-fourth ventricular spaces of the rat brain [16], these abstinence responses noted above indicate a low degree of dependence on opiates [7,16]. This was further evidenced by the fact that teeth chattering, escape responses and low incidence of wet shakes were observed only when the rats were challenged intraperitoneally with a fairly high dose (10 mg/kg) of naloxone. Additionally, the relative addiction liability of enkephalins appear to be lower than morphine [16].

More recently, attempts have been made to synthesize orally active, longer lasting peptides which are devoid of addiction liability [12] so that they can be used as effective analgesic drugs. Most of the newer synthetic enkephalins contain D-amino acids in position 2 and an amide function in the terminal aminoacid of the naturally occurring enkephalins. Our continuing efforts to find agents which either inhibit the development of tolerance and dependence on morphine [1, 4, 5, 6, 14, 15] or which attenuate the symptoms of morphine abstinence led us to assess the effects of two D-amino acid containing enkephalinamides in morphine dependent mice on abstinence syndrome induced by either withdrawing morphine or by using a narcotic antagonist, naloxone. Similar studies were carried out with morphine for comparison. The structure of naturally occurring enkephalins and D-amino acid containing enkephalins (which are presumably resistant to enzymatic degradation) are given below (Fig. 1).

METHOD

Prior to being used, male Swiss Webster mice weighing 25–30 g (Scientific Small Animals Inc., Arlington Heights, IL) were housed for at least four days in rooms with controlled temperature (23 ± 1°C), humidity (65 ± 2%) and artificial lightings (L 0600–1800 hr). The animals were allowed standard laboratory chow and tap water ad lib.

Mice were rendered dependent on morphine by the subcutaneous (SC) implantation of a specially formulated pellet for three days [1, 5, 13]. Each pellet contained 75 mg of morphine base. All pellet implantation and removal were done between 800 and 900 hr. A small incision in the back of the mouse was made for implantation and removal of the pellet and an anesthetic was not required. The pellet implantation procedure produces a high degree of dependence as evidenced by the intensity of both abrupt (body weight loss and hypothermia) and naloxone-induced (jumping response) abstinence response [1, 5, 13].

D-Ala^2-Met^5-enkephalinamide, D-Met^2-Pro^5-enkephalinamide (Bachem Chemical Co., CA) and morphine sulfate were dissolved in saline and administered intracerebroventricularly (ICV) in a volume of 5 μl. The injection technique has been described previously [3]. Brain sections taken after the injection of India ink revealed that the injection site was the third ventricle. Control animals received saline injections. Naloxone hydrochloride was dissolved in saline and administered SC in such a way that mice received 10 ml/kg.

Assessment of Abrupt and Antagonist-Induced Morphine Abstinence Responses

To study the effects of test drugs on abstinence, morphine pellets were removed three days after the implantation. Immediately after the pellet removal mice were divided into two groups; one group received the ICV injection of saline while the other received 1.75 μmole/kg of enkephalinamide or morphine sulfate. Body weight and rectal temperature of each mouse were measured over a 24-hr period at various time intervals using a digital thermistor thermometer as described before [5]. The data are expressed as mean ± SEM of the rectal temperature in each group of 8 mice. Similar treatment was carried out in separate groups of mice from which the pellets had been removed for 6 hr. The data were analyzed by the analysis of variance [9].

To study the effects of test drugs on naloxone-precipitated withdrawal the same procedure as above was used except that immediately after the enkephalinamide or morphine injection the animals were injected SC with naloxone (0.1 mg/kg). Their body weight and rectal temperature were measured as above. Eight mice were used in each group.

The effect of enkephalinamides or morphine was also studied on the naloxone-precipitated stereotyped jumping response. This response is a characteristic sign of morphine abstinence in rodents [2,13] and was used as a quantal response to determine the intensity of abstinence syndrome. In separate groups of morphine-dependent mice from which the morphine pellets had been removed for 6 hr, they were injected with saline ICV and immediately thereafter challenged with a SC injection of naloxone. The animals were placed on a circular platform and the percent of mice jumping off within a 15-min observation period was recorded. Three doses of naloxone were used to determine its ED_{50} (median effective dose to precipitate jumping) using 8 to 10 mice for each dose of naloxone. The dose-response curves were drawn by linear regression analysis and the naloxone ED_{50}, its 95% confidence limits and the statistical significance were determined by the method of Litchfield and Wilcoxon [11]. Similarly the naloxone ED_{50} was determined after administration of each dose of enkephalinamides or morphine. The ED_{50} values in the peptide or morphine treated mice were compared with those in the saline injected mice [11].

H-Tyr-Gly-Gly-Phe-Met-OH
(Methionine-enkephalin)

H-Tyr-Gly-Gly-Phe-Leu-OH
(Leucine-enkephalin)

H-Tyr-D-Ala-Gly-Phe-Met-NH_2
(D-Ala^2-Met^5-enkephalinamide)

H-Tyr-D-Met-Gly-Phe-Pro-NH_2
(D-Met^2-Pro^5-enkephalinamide)

FIG. 1. Structures of naturally occurring and synthetic enkephalins.

RESULTS

Withdrawal of morphine from morphine dependent mice produced hypothermia. Body temperature decreased from 35.5 to 33.8 at 5 to 6 hr after pellet removal and the decrease lasted for 12 hr. The body temperature returned to normal at 24 hr after withdrawal. This hypothermia was prevented for the entire 12 hr period by intracerebral administration of morphine, $F(1,9)=90.9$, $p<0.001$ (Fig. 2a), D-Ala^2-Met^5-enkephalinamide, $F(1,9)=79.4$, $p<0.001$ (Fig. 2b), and D-Met^2-Pro^5-enkephalinamide, $F(1,9)=76.2$, $p<0.001$ (Fig. 2c). Comparison of effects among the three groups indicated no significant differences in the intensity and duration of the change produced on body temperature. When naloxone was injected immediately after the pellet removal, hypothermia was also observed. Morphine prevented, $F(1,9)=80.9$, $p<0.001$, the hypothermia for 8 hr (Fig. 2d), whereas, the two enkephalinamides, D-Ala^2-Met^5-enkephalinamide, $F(1,9)=46.8$, $p<0.001$ (Fig. 2e) and D-Met^2-Pro^5-enkephalinamide, $F(1,9)=48.7$, $p<0.001$ (Fig. 2f) were effective for 12 hr. On molar basis D-Met^2-Pro^5-enkephalinamide was the most potent and morphine the least potent inhibitor of naloxone-precipitated withdrawal hypothermia.

Similar temperature changes occurred after abrupt withdrawal of morphine when morphine, $F(1,9)=88.7$, $p<0.001$ (Fig. 3a), D-Ala^2-Met^5-enkephalinamide, $F(1,9)=93.4$, $p<0.001$ (Fig. 3b), and D-Met^2-Pro^5-enkephalinamide, $F(1,9)=227.9$, $p<0.001$ (Fig. 3c) were administered 6 hr after morphine pellet removal. In these experiments, morphine, D-Ala^2-Met^5-enkephalinamide and D-Met^2-Pro^5-enkephalinamide reversed the abstinent hypothermia for 5, 8 and 12 hr, respectively. Similarly, naloxone precipitated was antagonized by morphine (Fig. 3d) $F(1,9)=89.6$, $p<0.001$, D-Ala^2-Met^5-enkephalinamide (Fig. 3e) $F(1,9)=99.0$, $p<0.001$, and D-Met^2-Pro^5-enkephalinamide, (Fig. 3f) $F(1,9)=56.8$, $p<0.001$.

Morphine as well as both enkephalinamides failed to modify the body weight loss which occurs after abrupt withdrawal of morphine. Maximum decrease (10%) in body

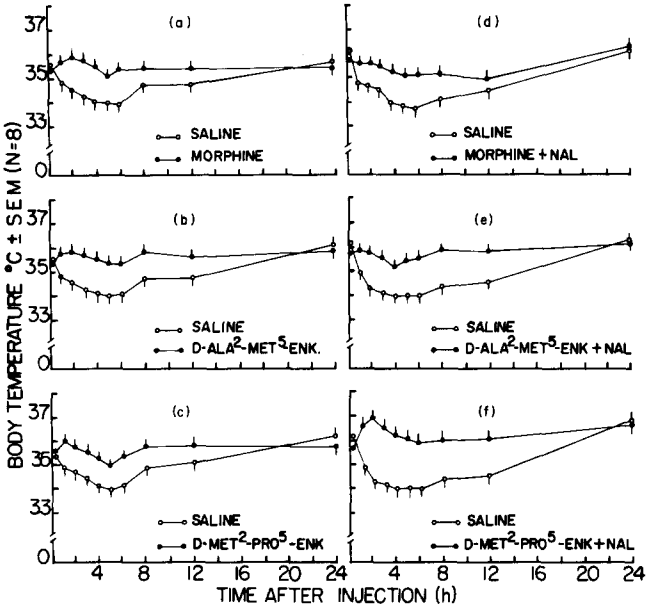

FIG. 2. Effect of morphine and enkephalinamides injected immediately after pellet removal on abrupt and naloxone-precipitated abstinent hypothermic response. Mice were rendered dependent by morphine pellet implantation for three days. The pellets were removed 72 hr after implantation, and immediately thereafter mice were injected ICV with saline or the test drug at 1.75 μmole/kg dose and the rectal temperature of each mouse was measured. Panels (a, b, and c)=abrupt abstinence. In precipitated abstinence (panels d, e, f), naloxone hydrochloride (NAL) (0.1 mg/kg SC) was injected immediately after saline or test drug injection. ENK=enkephalinamide. N=number of mice used in each group. The vertical bars represent the SEM.

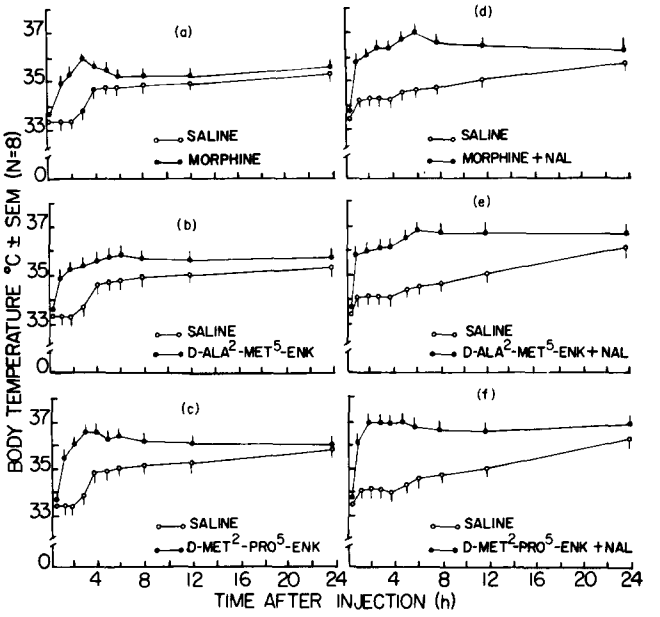

FIG. 3. Effect of morphine and enkephalinamides injected 6 hr after morphine pellet removal on abrupt and naloxone-precipitated abstinent hypothermic response. Mice were rendered dependent on morphine by pellet implantation for three days. The pellets were removed 72 hr after implantation. Six hours after the pellet removal mice were injected ICV with saline or the test drug at 1.75 μmole/kg and the rectal temperature of each mouse was measured. Panel a, b, and c represent temperatures during abrupt abstinence. In precipitated abstinence (panels d, e, f), naloxone hydrochloride (NAL) (0.1 mg/kg SC) was injected immediately after saline or test drug injection. ENK=enkephalinamide. N=number of mice used in each group. The vertical bars represent the SEM.

weight occurred 12 hr after morphine pellet removal and this decrease was similar in saline, morphine, and the two enkephalinamides injected morphine-dependent mice. These drugs did not modify the withdrawal defecation. Morphine-dependent mice (N=8) injected IC with saline and given a dose (25 μg/kg) of naloxone (SC) and placed on a circular platform had 30 fecal boli during the 15-min observation period. The response observed in morphine and enkephalinamide injected mice was not significantly different ($p>0.05$) than the saline injected controls.

Administration of morphine or either enkephalinamide reversed the stereotyped jumping syndrome. As shown in Fig. 4, morphine at 0.875 μmole/kg was ineffective, whereas, at 1.75 μmole/kg increased the naloxone ED_{50} by more than 4-fold over the ED_{50} in corresponding saline controls. D-Ala²-Met⁵-enkephalinamide increased the naloxone ED_{50} by 9, 11, and 85-fold at 0.875, 1.75, and 3.5 μmole/kg doses, respectively. Similarly D-Met²-Pro⁵-enkephalinamide increased the naloxone ED_{50} by 8, 91, and 216-fold over the saline injected control mice, at 0.875, 1.75, and 3.5 μmole/kg, respectively. When the effects of drugs on naloxone ED_{50} values were compared at 1.75 μmole/kg the drugs could be arranged in the following order of activity: D-Met²-Pro⁵-enkephalinamide>D-Ala²-Met⁵-enkephalinamide>morphine.

DISCUSSION

Intracerebral administration of morphine and two synthetic enkephalinamides to morphine dependent mice reversed both abrupt and naloxone-induced abstinence responses. Hypothermia, a characteristic narcotic abstinence response, was prevented by the test drugs when administered immediately after the morphine pellet was removed. Similar effects were observed on naloxone-precipitated abstinence hypothermia. Ferri *et al.* [8] have compared the effects of methionine-enkephalin (MEK) on core temperatures of morphine naive and morphine tolerant rats. They found that IP injection of MEK was without any effect on the core temperature in naive rats. However, intraventricular injection of MEK (100 μg) produced hyperthermia and higher doses (400 μg) produced hypothermia. Furthermore, the hypothermia produced by 400 μg was followed by hyperthermia. In rats rendered tolerant by daily injections of morphine, both 100 and 400 μg doses did not alter the temperature responses noted above. These studies indicate that enkephalins and morphine produced differential effects on body temperature which depends upon the dose of the drug used. When morphine or enkephalins were administered 6 hr after the pellet removal in this study; i.e., when the mice were already undergoing withdrawal and their body temperatures were below normal, a hyperthermic effect of long duration was observed. The prolonged prevention of hypothermia by enkephalinamides during morphine withdrawal indicates that these drugs are quite resistant to enzymatic degradation.

Morphine as well as the enkephalinamides also prevented

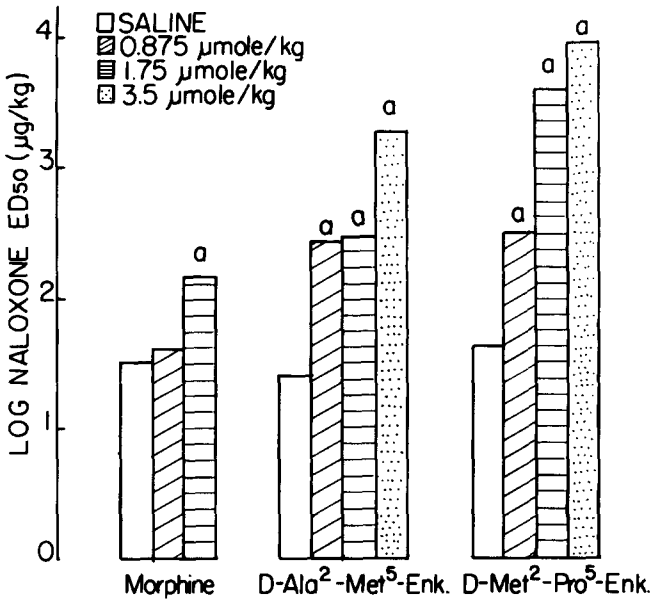

FIG. 4. Effect of morphine and enkephalinamides (ENK) on naloxone-precipitated stereotyped jumping response. Mice were rendered dependent by morphine pellet implantation for three days. The pellets were removed 72 hr after implantation and after an additional 6 hr mice were injected with saline or an appropriate dose of the test drugs. The naloxone ED_{50} for the jumping response was determined using three doses of naloxone. Eight to ten mice were used for each dose of naloxone. The 95% confidence limits of ED_{50} values are not shown for clarity. $^{a}p<0.05$ vs the saline injected controls.

the naloxone-precipitated jumping response in morphine dependent mice. The minimum dose of morphine which produced significant increase in naloxone ED_{50} was 1.75 μmole/kg. Both enkephalinamides appeared to be much more potent in inhibiting the jumping response, D-Met²-Pro⁵-enkephalinamide being much more potent than D-Ala²-Met⁵-enkephalinamide. On a molar basis D-Met²-Pro⁵-enkephalinamide and D-Ala²-Met⁵-enkephalinamide were 23 and 3 times more potent than morphine in this system. Previous studies from this laboratory [1,4] have indicated that the naturally occurring pentapeptides, MEK and leucine-enkephaline (LEK) are able to substitute for morphine and thus reverse the signs of morphine withdrawal. The dose-response relationship was difficult to establish for MEK and LEK, perhaps because of their rapid inactivation by the proteolytic enzymes in the brain.

Neither morphine nor the enkephalinamides prevented the body weight loss produced by morphine withdrawal or precipitated abstinence. Other peripheral signs, like defecation, diarrhea and micturition (data not shown) were similarly unaffected when these drugs were injected directly into the brain. These effects are consistent with our observations that the naturally occurring enkephalins are also unable to reverse many abstinence signs [1,4]. Consequently, the stereotyped jumping response and hypothermia during withdrawal seem to be centrally controlled, while the other signs such as defecation, diarrhea and micturition are not. This interpretation is further supported by other studies [16] which showed the absence of diarrhea even after precipitated withdrawal in rats made dependent on morphine by intracerebral infusion.

In summary, the present studies demonstrate that morphine as well as synthetic enkephalinamides injected ICV can reverse or prevent hypothermia and stereotyped jumping which are induced by morphine withdrawal in dependent mice. The peptides were actually more potent than morphine in preventing stereotyped jumping. The activity of naturally occurring pentapeptides is greatly enhanced when glycine in position 2 and methionine or leucine in position 5 are replaced by D-Met and Pro-NH_2, respectively. Among the three drugs studied, the order of activity in reversing morphine abstinence symptoms is as follows: D-Met²-Pro⁵-enkephalinamide>D-Ala²-Met⁵-enkephalinamide>morphine.

ACKNOWLEDGEMENTS

These studies were supported in part by a grant from the Illinois Department of Mental Health and Developmental Disabilities (No. 904-02). The author thanks George Matwyshyn for providing excellent technical assistance, Dr. Alan Rubin of Endo Laboratories, New York for kindly supplying the sample of naloxone hydrochloride, and Ms. Marie Zajac for her help in preparation of this manuscript.

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