Pharmacology Biochemistry & Behavior, Vol. 13, pp. 907–912. Printed in the U.S.A.

Naloxone-Like Actions of MIF-1 Do Not Require the Presence of the Pituitary

ABBA J. KASTIN,[1] CHERYL NISSEN, JAMES E. ZADINA,
ANDREW V. SCHALLY AND RUDOLPH H. EHRENSING*

Endocrinology Section of the Medical Service, Veterans Administration Medical Center
and
Department of Medicine, Tulane University School of Medicine, New Orleans, LA 70146
and
**Department of Psychiatry, Oschner Medical Institutions, New Orleans, LA 70121*

Received 7 October 1980

KASTIN, A. J., C. NISSEN, J. E. ZADINA, A. V. SCHALLY AND R. H. EHRENSING. *Naloxone-like actions of MIF-1 do not require the presence of the pituitary.* PHARMAC. BIOCHEM. BEHAV. 13(6) 907–912, 1980.—The actions of peripherally administered MIF-1 (Pro-Leu-Gly-NH_2) and naloxone in blocking the effects of morphine in the tail-flick test were measured across a wide range of five doses in hypophysectomized and intact mice. The presence of the pituitary gland failed to influence the response to MIF-1 or naloxone. Both hypophysectomized and intact mice were significantly affected by these two compounds at doses of 0.01, 0.1, 1.0, and 10.0 mg/kg IP. The greatest effect of MIF-1 occurred at 100 mg/kg, but naloxone was lethal at this dose. Preliminary experiments with other tests showed that at 10 mg/kg, naloxone, but not MIF-1, was effective in preventing the Straub-tail reflex and in precipitating withdrawal-jumping in mice implanted with morphine pellets. Only minimal activity was shown by MIF-1 in preventing blockade of electrically induced contractions of the guinea pig ileum by morphine. Neither compound was active in the frog-righting test. In summary, the results emphasize the differential actions of MIF-1 as an opiate antagonist and demonstrate that the pituitary is not required for their mediation.

Analgesia Naloxone Opiate Pituitary Peptide MIF-1 Guinea pig ileum Frog righting
Straub tail Withdrawal Tail-flick

MIF-1 (Pro-Leu-Gly-NH_2) recently has been shown to block the analgesia induced in mice by opiates as measured by the tail-flick test [4]. This hypothalamic peptide also has been known to exert other effects on the central nervous system (CNS) that have been termed "extra-pituitary" or "extra-endocrine" largely because of their activity in hypophysectomized animals [8, 9, 13]. It was decided, therefore, to examine the naloxone-like actions of several doses of MIF-1 in this task and determine whether the presence of the pituitary gland was essential for its effects.

METHOD

Tail-Flick

Male, white, ICR mice weighing about 20 g on arrival, were purchased from Harlan Sprague-Dawley (Madison, WI). They had free access to food and water for 2 days before testing. Hypophysectomized mice were fed special food (ICN Nutritional Biochemicals) and drank water containing 15% dextrose. They were tested 1 week after operation and completeness of hypophysectomy was checked visually after they were killed.

Throughout the two-hour period of testing, each mouse was kept in a 2.5 cm ID×5 cm Plexiglas tube. The mouse's tail was placed on a grooved tray so that it covered a small opening through which heat emanated from a nichrome heating wire. In this position, the tail blocked a photobeam that ran perpendicular, anterior, and superior to the heating element. A digital electronic timer was activated by turning on the heating unit and was deactivated when the mouse flicked its tail. Removal of the tail from the groove closed the photoelectric circuit and provided an accurate measurement of tail-flick latency.

All mice received an intraperitoneal (IP) injection of MIF-1, naloxone (Endo Labs), or diluent consisting of 0.01 M acetic acid in 0.9% NaCl. Ten minutes later, each mouse received an injection of morphine sulfate (15 mg/kg IP). Tail-flick latencies were measured immediately before the first injection and 15, 30, 60, 90, and 120 min afterwards. At each time, 4 trials were made, only the last 2 being recorded and their mean used for calculations. To prevent damage to the tail, each trial automatically ended at 10 seconds. At each dose level, 10 hypophysectomized and 10 intact mice received MIF-1 and an equal number of mice received naloxone. More than 50 additional mice were injected with diluent before the morphine. No mice were reused. Results were analyzed by analysis of variance. In those analyses

[1]Send reprint requests to, Dr. Abba J. Kastin, Endocrinology Section of the Medical Service, Veterans Administration Medical Center, 1601 Perdido St., New Orleans, LA 70146.

involving naloxone, all data from the 100 mg/kg dose were omitted.

Other Techniques

Straub-tail. As in the tail-flick test, male white ICR mice of the same weight and from the same supplier were injected with MIF-1, naloxone, or diluent 10 min before receiving morphine. Only one dose (10 mg/kg SC) of MIF-1 or naloxone was used, and the dose of morphine was 20 mg/kg. An additional group received pretreatment with diluent followed by a second injection of diluent instead of the morphine. Elevation of the proximal end of the tail was rated according to the technique of Kameyama *et al.* [3] in which no elevation (0°) received a score of 0, 1–44°=0.5, 45°=1.0, 46–89°=1.5, and 90°=2.0. Ratings were made immediately before any injection and 10, 20, 30, 40, 50, 60, and 120 min later. Twelve mice were tested in each group, half of them 1 to 4 hr into the light phase of a normal light:dark cycle (12:12 hr) and half of them 1 to 4 hr into the dark phase of a light: dark cycle that had been reversed 5 days earlier. Results were analyzed by analysis of variance followed by Duncan's Multiple Range Test.

Mouse-jumping withdrawal. A 75 mg pellet of morphine was implanted SC in the back of 30 g male Swiss Albino mice (Simonsen Labs) pretreated the previous day with three injections of morphine (10 mg/kg IP). After the pellets were in place 72 hours, groups of 4 mice were placed in a 2 liter glass beaker and jumping observed. Any mouse jumping to the top of the beaker more than 5 times within 30 minutes was excluded. Mice were then randomly divided into groups of 5 to receive a single IP injection of 1 of 3 solutions: MIF-1 (10 mg/kg), naloxone (10 mg/kg), or diluent. Mice were replaced in the 2 liter beakers and the number of jumps to the top of the beaker was recorded for an hour at 15 min intervals. Results were analyzed by analysis of variance followed by Duncan's Multiple Range Test.

Frog-righting. Male *Rana pipiens* frogs (Lemberger, Ltd.) weighing about 35 g were injected in the dorsal lymph sac with 100 mg/kg apomorphine. An hour later, each frog received a single dose of one of the following solutions: MIF-1, naloxone, or amitriptyline. MIF-1 and naloxone were tested at 0.01, 0.1, 1.0, 10, and 30 mg/kg, 12 different frogs at each dose. The amitriptyline used as a control was tested at the single dose of 14 mg/kg. Fifteen minutes after injection of the MIF-1, naloxone, or amitriptyline, the righting reflex was assessed by placing the frog on its back 10 successive times. Loss of righting was indicated by a failure of the frog to assume its normal position within 15 sec.

Guinea-pig ileum. The terminal 2 cm of ileum was obtained from male albino guinea pigs (HLA Hartley) weighing about 200 g. After the contents were gently expressed from the lumen, an individual ileum was suspended vertically between platinum wire electrodes in a 5 ml bath of Krebs Ringer's bicarbonate glucose (KRBG), maintained at 37°C, through which 5% CO_2–95% O_2 was gently bubbled. The tissue was subjected to 0.5 g applied tension and allowed to equilibrate 45 min before use. Electrical stimulation of the ileum was provided through an isolation unit (Grass SIU 478A) by a Grass S-4 stimulator delivering rectilinear pulses of 1.0 msec duration at a frequency of 0.1 Hz and a voltage setting indicating 3 times maximum (about 80 V). Contractions were isometrically recorded by a force displacement transducer (Grass Model FT03C) on a polygraph (Grass Model 7). Inhibition (ED50) of the contractions was caused by addition of 5×10^{-8}M morphine. MIF-1 was added 1 minute before or after the morphine in doses ranging from 10^{-8}M to 10^{-4}M.

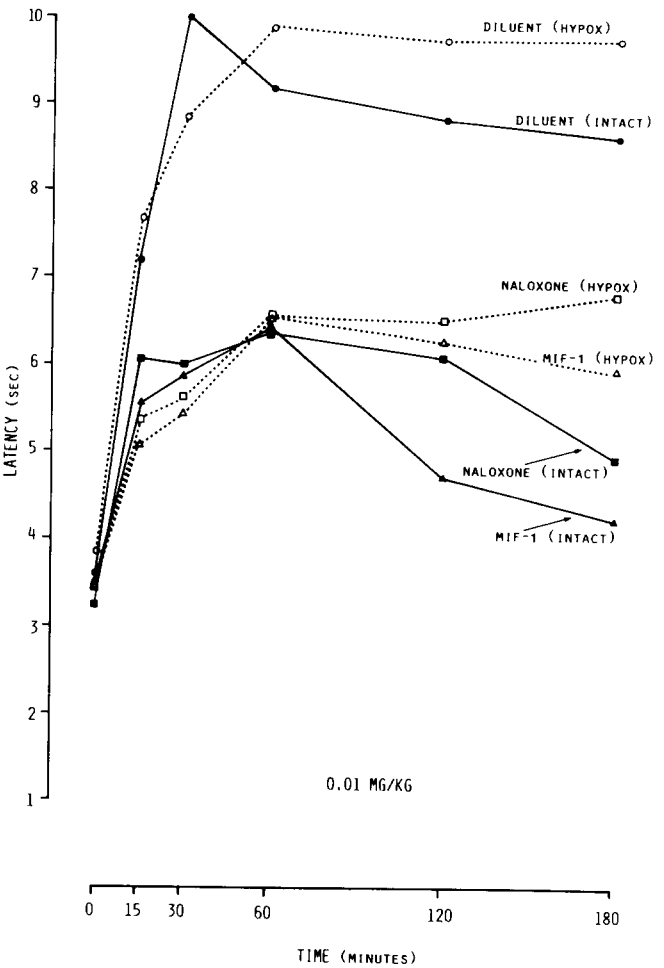

FIG. 1. Reversal of blockade of tail-flick response to morphine by pretreatment with 0.01 mg/kg IP of MIF-1 or naloxone in hypophysectomized and intact mice.

RESULTS

Tail-Flick

Both naloxone and MIF-1 were effective in blocking the morphine-induced increase in tail-flick latency. The main effect of material injected (naloxone, MIF-1, or diluent) was significant, $F(2,194)=117.99$, $p<0.001$, and the scores for both MIF-1 and naloxone-treated mice were significantly lower than those for diluent-injected controls. Even at the lowest dose tested (0.01 mg/kg), both MIF-1 and naloxone significantly reduced latency to response below that of the diluent group, $F(1,34)=17.14$, $p<0.001$; $F(1,34)=28.42$, $p<0.001$. Reversal of the effects of morphine by naloxone, however, was greater than for MIF-1, $F(1,157)=7.01$, $p<0.01$.

The morphine-blocking action of naloxone and MIF-1 generally followed a conventional dose-response pattern (Figs. 1–5). The main effect of dose was significant, $F(3,157)=8.43$, $p<0.001$. However, the injected material × dose × trials interaction was also significant, $F(15,785)=$

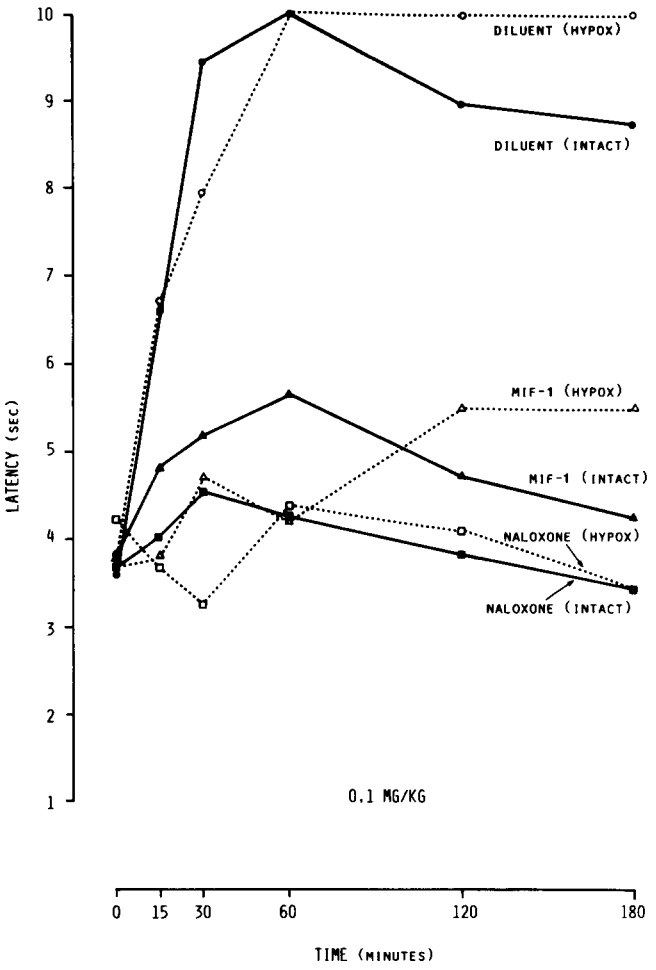

FIG. 2. Reversal of blockade of tail-flick response to morphine by pretreatment with 0.1 mg/kg IP of MIF-1 or naloxone in hypophysectomized and intact mice.

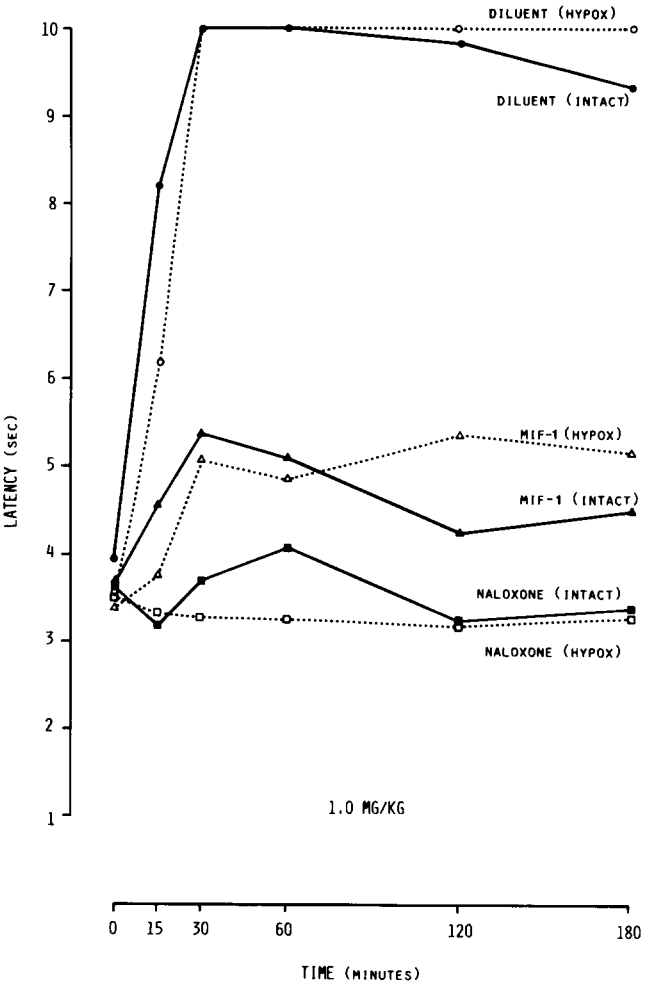

FIG. 3. Reversal of blockade of tail-flick response to morphine by pretreatment with 1.0 mg/kg IP of MIF-1 or naloxone in hypophysectomized and intact mice.

1.74, $p<0.05$, reflecting the stronger effect of dose for naloxone than for MIF-1, especially at the later time periods. This is further illustrated by separate analysis of the MIF-1 data showing that the effect of dose was significant when the 100 mg/kg dose was included in the analysis, $F(4,142)=2.8$, $p<0.05$, but not when the range included only the 0.01–10 mg/kg dosages. Although MIF-1 was maximally effective and showed no toxicity at 100 mg/kg, naloxone was lethal within ten minutes at this dosage.

Hypophysectomy did not change the baseline tail-flick response or the blockade of morphine-induced analgesia by MIF-1 or naloxone. The main effect of surgery was not significant ($p>0.05$), and basal latencies at time 0 showed no significant differences due to any treatments and no significant interactions. However, the latencies found in hypophysectomized mice were higher at the later time trials in all injection groups. This was supported by the trials × surgery interaction, $F(5,970)=6.22$, $p<0.001$, and by a significant effect of surgery at the 180 minute time period, $F(1,194)=5.96$, $p<0.05$. It was especially clear at the 0.01 mg/kg dose (Fig. 1), but was not dependent on dose or injected substance since no other interactions with surgery were significant.

Other Techniques

Straub-tail. Analysis of variance revealed a significant main effect of treatment, $F(3,40)=21.75$, $p<0.01$, trial, $F(5,200)=5.35$, $p<0.001$, and treatment by trial, $F(15,200)=2.51$, $p<0.01$. At 30 minutes, the time of the greatest differences, Duncan's Multiple Range Test showed that during either light cycle the scores obtained with mice receiving naloxone were significantly ($p<0.01$) lower than those from animals injected with MIF-1 or diluent. The effect of pretreatment with MIF-1 could not be distinguished from that of pretreatment with diluent in the morphine-injected

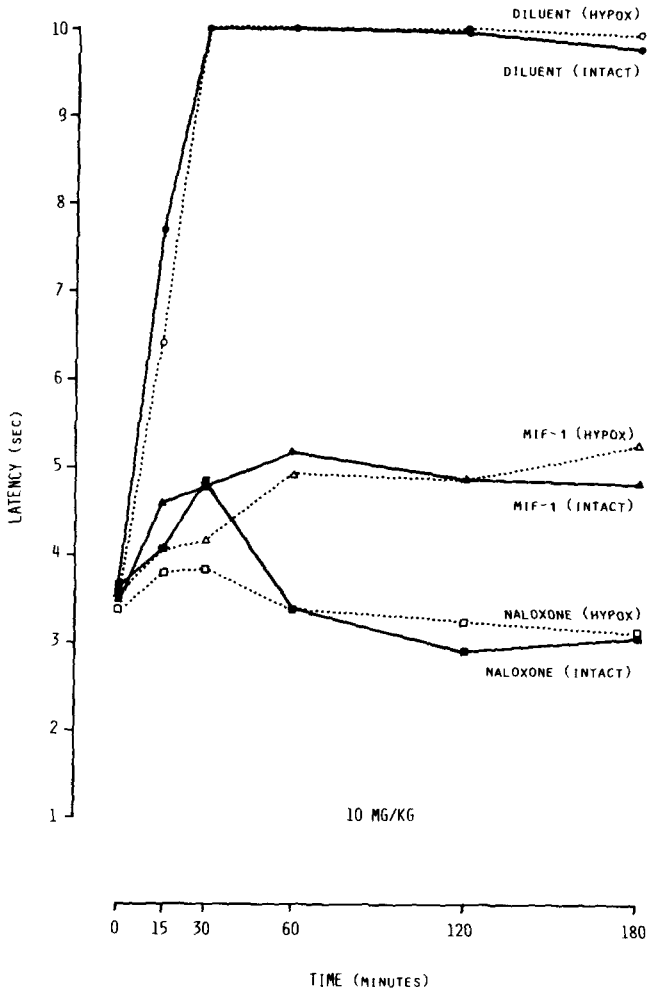

FIG. 4. Reversal of blockade of tail-flick response to morphine by pretreatment with 10.0 mg/kg IP of MIF-1 or naloxone in hypophysectomized and intact mice.

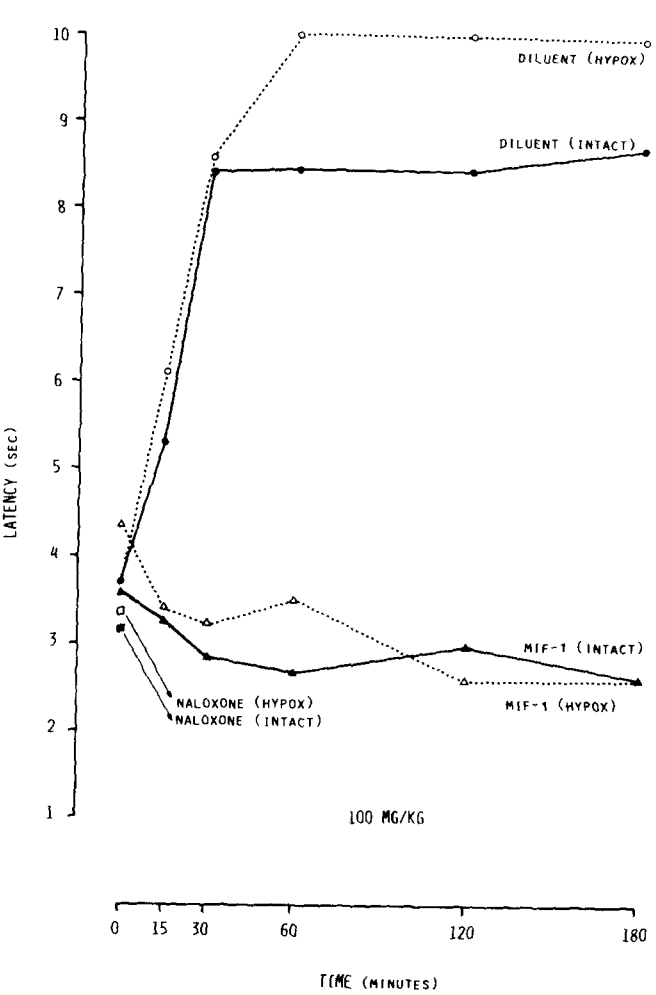

FIG. 5. Reversal of blockade of tail-flick response to morphine by pretreatment with 100 mg/kg IP of MIF-1. Naloxone was lethal at this dose.

mice under either lighting schedule. Pretreatment of morphine-injected mice with naloxone completely eliminated the Straub-tail reaction so that they could not be distinguished from mice receiving only diluent without morphine.

Withdrawal jumping. As expected, the naloxone-precipitated withdrawal in mice implanted for 3 days with morphine resulted in an increase in jumping behavior. Analysis of variance revealed a significant main effect of treatment, $F(2,7)=7.18$, $p<0.05$, trial, $F(3,21)=6.46$, $p<0.01$, and a treatment by trial interaction, $F(6,21)=6.35$, $p<0.001$. Duncan's Multiple Range Test showed that at each time, naloxone caused significantly ($p<0.05$ at 15 and 30 minutes; $p<0.01$ at 45 and 60 minutes) more jumps than did either MIF-1 or diluent. There was no significant difference between the effects of diluent and MIF-1, although the experimenter was usually able to predict which mice received the MIF-1 by their greater activity. This activity, however, was not sufficient to enable the mice receiving MIF-1 to meet the jumping criterion for significant withdrawal.

Frog-righting. Analysis of variance revealed that none of the 5 doses of MIF-1 or naloxone caused a significant alteration in the righting reflex in frogs pretreated with apomorphine. This is in contrast to the marked ($p<0.001$) depression of righting caused by injection of amitriptyline. The same dose of amitriptyline in the absence of apomorphine was without effect.

Guinea-pig ileum. Pretreatment with 5×10^{-5} M MIF-1 reduced from 50% to 42% the inhibition of electrically induced contractions of the guinea pig ileum caused by morphine. This slight effect was not observed when the MIF-1 was added after rather than before the morphine. In contrast to MIF-1, naloxone completely blocked the actions of morphine, even when added at smaller doses.

DISCUSSION

The absence of the pituitary gland did not influence baseline responsiveness to the tail-heating apparatus; the response time for removal of the tail before the injection of 15 mg/kg morphine (time 0) was the same in intact and hypophysectomized mice.

The effect of hypophysectomy on responsiveness to morphine, however, was not as clear. In a preliminary study, we found that it was necessary to use the 15 mg/kg dose of morphine to achieve maximal analgesia in hypophysectomized mice as compared with only 10 mg/kg in intact mice. These results indicated a decreased sensitivity to morphine after hypophysectomy. In the present study, hypophysectomy tended to increase the duration of analgesia regardless of material injected; this resulted in greater tail-flick latencies at 180 minutes. In a study not using morphine, Grevert *et al.* [2] found that the presence of the pituitary was necessary for naloxone to reduce latency to escape from the hotplate. The present study indicates that blockade of morphine-induced analgesia is not dependent on the pituitary. The differences in tail-flick latencies between intact and hypophysectomized animals in the groups given morphine plus an opiate antagonist were not altered beyond changes seen in the morphine plus diluent group. Thus, although hypophysectomy may change sensitivity to morphine, it did not appear to affect the blockade of morphine-induced analgesia by either naloxone or MIF-1.

The lack of mediation by the pituitary of the effects of MIF-1 in the tail-flick test might have been expected from other tests. It was the activity of MIF-1 in the dopa-potantiation test in hypophysectomized mice that was responsible for the introduction of the concept of the extra-pituitary or extra-endocrine effects of brain peptides [8]. Similarly, the pituitary gland was unnecessary for the reversal by MIF-1 of oxotremorine-induced tremors [9] and chlorpromazine-induced hypothermia [13].

The necessity for preventing burning of the tail by imposition of a 10 second cut-off time restricted the available response range and contributed to the tendency for an all-or-none effect of the MIF-1 and naloxone. Thus, increasing the dose of antagonist tended to increase the probability of a full response rather than a gradual increase in intensity of response. The close molecular weights of the naloxone (327) and MIF-1 (284) makes it unlikely that injection of equimolar amounts would have changed interpretations about the relative potencies of the compounds. Although the low dose of 0.01 mg/kg appeared less effective than higher doses for both opiate antagonists, their activities were still significant. The high dose of 100 mg/kg was toxic for naloxone but not for MIF-1; MIF-1 was the most active at this dose. An inverted U-shaped curve has been frequently described for the non-opiate actions of peripherally administered MIF-1 [5] but was not observed for its actions as an opiate antagonist.

Disagreement as to whether MIF-1 facilitates or blocks the action of opiates has been raised with regard to opiate tolerance. Van Ree and DeWied [11] and Szekely *et al.* [10] reported a facilitation of opiate tolerance by MIF-1 while Walter *et al.* [12] and Bhargava *et al.* [1] found that the peptide blocked tolerance or dependence. In our withdrawal-jumping study, we observed, as Bhargava *et al.* [1] reported while our study was in progress, that MIF-1 did not reverse physical tolerance or dependence already induced by implantation of a morphine pellet for 72 hours. However, Bhargava *et al.* [1] found that MIF-1 did inhibit the development of this tolerance or dependence if given before the morphine. It might be expected from these findings that the naloxone-like actions of MIF-1 require administration before rather than after an opiate. This may not be true for acute opiate effects. Although we injected the MIF-1 before morphine in the tail-flick test in the present study, preliminary results from a similar study [4] indicated some blockade of analgesia when MIF-1 was given 10 minutes after the morphine. By contrast, we observed that injection of MIF-1 10 minutes before a slightly larger dose of morphine (20 mg/kg) did not reverse the Straub-tail reflex, even though naloxone was effective.

Unlike the actions of naloxone, MIF-1 did not reverse the inhibition of electrically-induced contractions of the *vas deferens* [4] and showed only weak activity in the guinea pig ileum assay. Although this suggests a greater effect of MIF-1 on mu than delta opiate receptors, the effect appears too small to explain the results of the tail-flick test. Thus, while actions of MIF may be partially mediated by mu receptors, other opiate or non-opiate receptors may also be involved.

The lack of effect of MIF-1 in altering the righting reflex of frogs also could be considered to constitute some weak evidence against effects of MIF-1 on serotonergic mechanisms [6]. In mice, MIF-1 had been previously shown to be inactive in the serotonin-potentiation test [7].

Thus, although MIF-1 can act like naloxone in a number of situations, many differences exist. In the tail-flick test, the effects of the two structurally different compounds are very similar and are not mediated by the pituitary gland.

ACKNOWLEDGEMENTS

We appreciate the help of the following students: Jack Lehman in the mouse-jumping withdrawal test, Shelley Threefoot in the frog-righting test, Ted Gilman and Michael Jemison in the Straub tail test, Claudia Robin in the tail-flick test, and Michael Mauk in the statistical analyses. Expert secretarial help was provided by Mrs. Jeri Liles. The studies were supported in part by the Medical Research Service of the Veterans Administration and NIH (NS 07664).

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