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THE EFFECT OF MELANOTROPHIN RELEASE INHIBITING FACTOR (MIF) AND CYCLO (LEU-GLY) ON THE TOLERANCE TO MORPHINE-INDUCED ANTINOCICEPTION IN THE RAT: A DOSE-RESPONSE STUDY

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1 The effects of melanotrophin release inhibiting factor (MIF) and its cyclic analogue cyclo (Leu-Gly) on tolerance to the analgesic effect of morphine were studied in male Sprague-Dawley rats.

2 Tolerance to morphine was induced by implantation of four morphine pellets (each containing 75 mg of morphine free base) during a 3 day period.

3 Daily subcutaneous administration of MIF and cyclo (Leu-Gly) before and during the morphine pellet implantation inhibited the development of tolerance to morphine analgesia. The minimum daily dose of the peptides required to produce a significant effect was 0.5 mg/kg.

4 The effects of single injections of MIF and cyclo (Leu-Gly) on morphine tolerance revealed that the minimum doses of cyclo (Leu-Gly) and MIF to inhibit morphine tolerance were 4 and 8 mg/kg, respectively.

5 Chronic treatment with morphine resulted in an enhanced hypothermic response to a dopamine agonist, apomorphine. The enhancement of this response was blocked by both MIF and cyclo (Leu-Gly) in doses that inhibited morphine tolerance.

6 It is concluded that MIF and cyclo (Leu-Gly) block the development of analgesic tolerance as well as dopamine receptor hypersensitivity induced by chronic morphine treatment and the two phenomena may be interrelated.

Introduction

Certain neurohypophysial hormones and analogues are known to have profound effects on the central nervous system (CNS). For instance, vasopressin and its analogues were shown to facilitate active avoidance behaviour in hypophysectomized (Bohus, Gispen & de Wied, 1973) and in normal rats (King & de Wied, 1974). These peptides, after systemic or intercerebral administration, inhibit extinction of active and passive avoidance responses in rats (de Wied, Bohus, Urban, van Wimersma Greidanus & Gispen, 1975) and attenuate the amnesia caused by puromycin in mice (Flexner, Flexner, Hoffman & Walter, 1977). The above studies indicate that these peptides may be involved in learning and memory.

The development of tolerances to opiates has been regarded as a form of learning or memory (Cohen, Keats, Krivoy & Unger, 1965). With this background information, Krivoy, Zimmerman & Lande (1974) reported that desglycineamide^9-lysine vasopressin (DG-LVP), an analogue of vasopressin which has behavioural effects essentially similar to those of vasopressin but which has little effect on the endocrine system (de Wied, Greven, Lande & Witter, 1972), facilitates the development of tolerance to the analgesic effect of morphine in mice. Evidence for the possible involvement of neurohypophysial hormones in the modification of opiate tolerance was further provided by the studies of de Wied & Gispen (1976) in Brattleboro rats with hereditary diabetes insipidus which lack the ability to synthesize vasopressin. These animals exhibited a delayed development of tolerance to morphine analgesia unless maintained on arginine vasopressin or DG-LVP. Additional studies (van Ree & de Wied, 1976) indicated that not only DG-AVP, but oxytocin and its C-terminal tripeptide prolylleucyl-glycinamide (MIF) and cyclo (Leu-Gly) facilitated morphine dependence (as measured by the loss of body weight following naloxone administration to dependent rats) as well as development of tolerance to the analgesic effect of morphine. Other studies, however, indicate that oxytocin and vasopressin (Schmidt, Holaday, Loh & Way, 1978) and

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cyclo (Leu-Gly) (Bhargava, 1980a) do not facilitate morphine tolerance or dependence in rats.

Previous studies have demonstrated that an analogue of MIF, Z-Pro-D-Leu, (Walter, Ritzmann, Bhargava, Rainbow, Flexner & Krivoy, 1978), MIF and cyclo (Leu-Gly) (Walter, Ritzmann, Bhargava & Flexner, 1979; Bhargava, Walter & Ritzmann, 1980) inhibit the development of tolerance to and physical dependence on morphine in mice. Additionally, it was found that cyclo (Leu-Gly) also inhibited morphine tolerance in the rat (Bhargava, 1980b).

In this paper, the effect of single and multiple injections of MIF and cyclo (Leu-Gly) on the development of tolerance to the analgesic effect of morphine in the rat is described. A dose-response relationship with both the linear as well as the cyclic peptide has also been carried out in order to determine the minimum dose of the peptide necessary for the inhibition of morphine tolerance. Finally the effects of smallest doses of the peptides which inhibited morphine tolerance have been determined on the supersensitivity of brain dopamine receptors induced by chronic administration of morphine.

Methods

Animals

Male Sprague-Dawley rats weighing 200 to 250 g obtained from King Animal Laboratories, Oregon, Wis., were housed 3 to a cage in a room with controlled temperature (23 ± 1°C), humidity (65 ± 2%) and 12 h dark-light cycle (light from 06 h 00 min to 18 h 00 min). Food and water were continously available. Rats were housed under these conditions for at least four days before being used.

Chemicals

MIF was a gift from the Abbott Laboratories, N. Chicago, Illinois through the courtesy of Dr A.O. Geiszler. Cyclo (Leu-Gly) was synthesized according to the method of Fischer (1906). Apomorphine hydrochloride was purchased from Sigma Chemical Co., St. Louis, Missouri. MIF and cyclo (Leu-Gly) were dissolved in water and injected subcutaneously. Morphine sulphate was dissolved in saline and injected intraperitoneally (i.p.). Apomorphine was dissolved in saline containing 0.01% ascorbic acid and injected intraperitoneally. The injection volume for each drug was 1 ml/kg.

Effect of MIF and cyclo (Leu-Gly) on morphine tolerance

Tolerance to morphine in the rat was induced by subcutaneous implantation of four morphine pellets during a 3-day period as described previously (Bhargava, 1977, 1978, 1979). Each pellet contained 75 mg of morphine free base. The control rats were implanted with an equivalent number of placebo pellets which contained the excipients but no drug.

The effects of both multiple (daily injections for three days) and single injections (2 h before pellet implantation) of the peptides on morphine tolerance were determined. To study the effect of MIF or cyclo (Leu-Gly), rats were injected in the morning with either vehicle (water) or an appropriate dose of the peptide. Two hours later the rats were divided into two subgroups. Rats in one subgroup were implanted with a placebo pellet, while those in the other subgroup received a morphine pellet. The pellets were implanted under light ether anaesthesia. On the same day at 16 h 30 min the rats were implanted with one more pellet (placebo or morphine) in their respective groups as described before (Bhargava, 1980b). On the morning of the second day the injections of vehicle or the peptides were repeated and at 16 h 30 min two placebo or morphine pellets were implanted in their respective groups. On day 3, the injections of vehicle or the peptide were given for the third time. In studies involving single injections of peptides, animals were injected 2 h before implantation of the first pellet. Three more morphine pellets were implanted as described above. The pellets were removed under light ether anaesthesia from all the rats 70 h after the first implantation. Six hours after pellet removal, the analgesic response to morphine was determined using a tail-flick apparatus. The tail-flick latencies to thermal stimulation were determined before and at 30 min after a test dose of morphine sulphate. The light source was adjusted in such a way that the premorphine or baseline tail-flick reaction time was 2.4 ± 0.2 (s.e.) s. A value of 20 s was used as the cut-off point to avoid damage to the tail. The analgesic response for each rat was calculated according to the following formula:

$$\% \text{ analgesia} = \frac{T_t - T_o \times 100}{20 - T_o}$$

where T_o is the base line tail-flick reaction time (in s) and T_t is the reaction time at t min after morphine injection. The data are expressed as mean % analgesic response ± s.e. mean. The difference in the vehicle and peptide-treated groups were analyzed by the paired or unpaired Student's *t* test.

Effect of MIF and cyclo (Leu-Gly) on dopamine receptor sensitivity

Dopamine receptor sensitivity was determined by measuring the hypothermic response induced by the dopamine agonist, apomorphine. Additional groups of rats were treated with vehicle, MIF (8 mg/kg, s.c.)

Table 1 Effect of multiple injections of cyclo (Leu-Gly) on the development of tolerance to the analgesic effect of morphine

Treatment[a]	*Dose of cyclo (Leu-Gly)* (mg/kg)	*Dose of morphine* (mg/kg)	*% analgesia at 30 min after morphine injection* (mean ± s.e. mean, $n = 8$)
Water + placebo	–	1	20.3 ± 5.5
Water + morphine	–	6	8.6 ± 4.0
Cyclo (Leu-Gly) + morphine	0.05	6	12.5 ± 3.8
Cyclo (Leu-Gly) + morphine	0.10	6	15.1 ± 3.5
Cyclo (Leu-Gly) + morphine	0.50	6	20.5 ± 5.6*
Cyclo (Leu-Gly) + morphine	1.00	6	27.8 ± 5.5*

[a] Rats were injected with water or an appropriate dose of cyclo (Leu-Gly). They were then implanted subcutaneously, with 4 morphine pellets over a 3 day period. Water and cyclo (Leu-Gly) injections were repeated twice more, 24 h apart. The pellets were removed 70 h after the implantation. Six hours later, the analgesic response to morphine was determined. One group of rats was injected with saline and implanted with 4 placebo pellets.
* $P<0.05$ vs water + morphine group.

or cyclo (Leu-Gly) (4 mg/kg s.c.). They were then divided into 2 groups, one being implanted with four placebo pellets, and the other with four morphine pellets as described above. The doses of the peptides used were based on single dose studies which indicated that they blocked the tolerance to the analgesic effect of morphine. The pellets were removed 70 h after the first implantation. Dopamine receptor sensitivity was measured after an additional 24 h. Rats in each group were injected intraperitoneally with apomorphine HCl (2 mg/kg). Body temperature was measured by using a rectal probe and a telethermometer (Yellow Spring Instrument Co., Yellow Springs, Ohio). The first reading was taken just before apomorphine injection and was repeated at 20 min after the injection. The data are expressed as rectal temperature (°C), mean ± s.e.mean. Six rats were used for each group. The data were analyzed by Student's unpaired *t* test.

Results

Effect of multiple injections of cyclo (Leu-Gly) and MIF on the development of analgesic tolerance to morphine

Daily injections of cyclo (Leu-Gly) during the time of morphine pellet implantation inhibited the development of tolerance to the analgesic effects of morphine. It was previously shown that administration of cyclo (Leu-Gly) to rats implanted with placebo pellets alters neither base line tail-flick reaction time nor morphine-induced analgesia (Bhargava, 1980b). The base line reaction to thermal stimulation was also not altered in rats implanted with morphine pellets and remained at 2.4 ± 0.2 (s.e.) s. The effect of various doses of cyclo (Leu-Gly) on morphine tolerance is shown in Table 1. A dose of morphine (1 mg/kg) produced 20% analgesia at 30 min after its

Table 2 Effect of multiple injections of MIF on tolerance to the analgesic effect of morphine

Treatment[a]	*Dose of MIF* (mg/kg s.c.)	*Dose of morphine* (mg/kg i.p.)	*n*	*% analgesia at 30 min after morphine injection* (mean ± s.e.mean)
Vehicle + placebo	–	2	6	49.7 ± 15.0
MIF + placebo	4	2	6	31.3 ± 7.8
Vehicle + morphine	–	8	7	36.5 ± 14.1
MIF + morphine	2	8	7	74.0 ± 16.9*
MIF + morphine	4	8	7	64.9 ± 10.0*

[a] Rats were injected with vehicle (water) or an appropriate dose of MIF. They were then implanted with 4 morphine pellets over a 3 day period. Vehicle and MIF injections were repeated twice more, 24 h apart. The pellets were removed 70 h after the implantation. Six hours later, the analgesic response to morphine was determined. Similar studies were carried out with rats implanted with placebo pellets which were injected with vehicle and MIF.
* $P<0.05$ vs. vehicle + morphine-treated groups.

administration in rats implanted with placebo pellets. Morphine (6 mg/kg) administered to rats implanted with morphine pellets produced only 9% analgesia at 30 min after its administration indicating that tolerance to morphine analgesia had developed. In rats treated with cyclo (Leu-Gly) in doses of 0.05 and 0.1 mg/kg daily for three days and implanted with morphine pellets, the tolerance to morphine analgesia was not affected. Even though the rats treated with cyclo (Leu-Gly) showed greater analgesia as compared to vehicle treated morphine-tolerant rats, statistical significance was not observed. Multiple injections of cyclo (Leu-Gly) in doses of 0.5 and 1.0 mg/kg significantly inhibited the development of tolerance to morphine. The analgesic response observed at 30 min following morphine injection in rats treated with the 0.5 and 1.0 mg/kg doses was 21 and 28% as compared to 8.6% in vehicle injected controls (Table 1).

Multiple administration of MIF also inhibited the development of tolerance to the analgesic effect of morphine. Table 2 shows the effect of two doses of MIF on morphine tolerance. The effect of the higher dose (4 mg/kg) administered repeatedly, on morphine analgesia in placebo pelleted rats was determined. At 30 min after the morphine (2 mg/kg) injection, the analgesic response in vehicle- and MIF-treated rats was similar. In morphine-tolerant rats, morphine sulphate (8 mg/kg) produced approximately the same degree of analgesia as that produced by 2 mg/kg of morphine in rats implanted with placebo pellets, again indicating the development of morphine tolerance. Rats injected daily with 2 and 4 mg/kg of MIF showed significantly greater ($P<0.05$) analgesic response to morphine as compared to morphine-tolerant rats treated with vehicle.

Since both the 2 and 4 mg/kg doses of MIF effectively inhibited the development of morphine tolerance, the effect of lower doses of MIF was determined in order to find the minimum effective dose. The effect of daily administration of MIF in doses of 0.25, 0.5 and 1.0 mg/kg on morphine tolerance is shown in Table 3. As shown before, MIF administration did not alter morphine analgesia in rats implanted with placebo pellets. Rats which were treated with vehicle and implanted with morphine pellets showed a 11.5% analgesia at 30 min after the administration of morphine (8 mg/kg). Daily injections of 0.25 mg/kg of MIF were ineffective in altering morphine tolerance since the analgesic response to morphine in MIF- and vehicle-treated morphine tolerant rats was similar. MIF in doses of 0.5 and 1.0 mg/kg was effective in inhibiting tolerance to the analgesic effect of morphine as indicated by greater analgesia in MIF-treated rats than in vehicle-treated rats.

Effect of single injection of cyclo (Leu-Gly) and MIF on the development of tolerance to analgesic effect of morphine

Administration of a single dose of cyclo (Leu-Gly) or MIF also inhibited the development of morphine tolerance (Table 4). Administration of cyclo (Leu-Gly) or MIF at 4 mg/kg dose did not alter morphine analgesia in rats with placebo pellets. As noted above, approximately 4 fold tolerance to morphine developed as a result of pellet implantation since the response to 8 mg/kg of morphine in rats with morphine pellets and to 2 mg/kg of morphine in rats with placebo pellets were similar. Cyclo (Leu-Gly) (2 mg/kg) and MIF (4 mg/kg) failed to affect morphine tolerance development. However, in rats treated with cyclo (Leu-Gly) in doses of 4 and 8 mg/kg and MIF in a dose of 8 mg/kg, significantly greater ($P<0.05$) analgesic effect was produced than in the corresponding vehicle-injected controls.

Table 3 Effect of multiple injections of MIF on tolerance to the analgesic effect of morphine

Treatment[a]	*Dose of MIF* (mg/kg s.c.)	*Dose of morphine* (mg/kg i.p.)	*n*	*% analgesia at 30 min after morphine injection* (mean ± s.e.mean)
Vehicle + placebo	–	2	4	12.5 ± 1.8
MIF + placebo	1	2	4	8.0 ± 3.3
Vehicle + morphine	–	8	6	11.5 ± 3.5
MIF + morphine	0.25	8	8	10.9 ± 1.7
MIF + morphine	0.50	8	7	19.9 ± 3.3*
MIF + morphine	1.0	8	6	25.3 ± 4.4*

[a]Rats were injected with vehicle (water) or an appropriate dose of MIF and then implanted with placebo pellets as described in the footnote to Table 2. Six hours after the pellet removal, the analgesic response to morphine was determined in each group of rats.

* $P<0.05$ vs. vehicle + morphine-treated group.

Table 4 Effect of single administration of cyclo (Leu-Gly) and MIF on the development of tolerance to the analgesic effect of morphine

Treatment[a]	*Dose of peptide* (mg/kg s.c.)	*Dose of morphine* (mg/kg i.p.)	*n*	*% analgesia at 30 min after morphine injection* (mean ± s.e.mean)
With placebo pellets				
Water	–	2	4	40.8 ± 11.1
Cyclo (Leu-Gly)	4	2	4	35.3 ±5.2
MIF	4	2	4	31.3 ± 9.8
With morphine pellets				
Water	–	8	8	40.9 ± 5.0
Cyclo (Leu-Gly)	2	8	8	35.0 ± 5.1
Cyclo (Leu-Gly)	4	8	8	50.3 ± 4.3*
Cyclo (Leu-Gly)	8	8	8	52.0 ± 3.6*
MIF	4	8	8	30.8 ± 4.8
MIF	8	8	8	49.5 ± 3.6*

[a]Rats were injected with water, cyclo (Leu-Gly) or MIF. Two hours later rats were implanted with four placebo or morphine pellets as described in the text. Six hours after the pellet removal analgesic response to an appropriate dose of morphine was determined.
* $P<0.05$ vs. the corresponding water-treated group.

Effect of cyclo (Leu-Gly) and MIF on apomorphine-induced hypothermia in rats implanted with placebo or morphine pellets

In rats from which placebo pellets had been withdrawn for 24 h, administration of apomorphine (2 mg/kg) produced a hypothermic response. A significant decrease (1.1°C) in body temperature was noted at 20 min after apomorphine injection. A similar hypothermic effect was seen in rats with placebo pellets pretreated with single injections of cyclo (Leu-Gly) (4 mg/kg) and MIF (8 mg/kg). These doses of cyclo (Leu/Gly) and MIF were shown above to inhibit significantly the development of tolerance to the analgesic effect of morphine (Table 5). The body temperature before injection of apomorphine of placebo and morphine pelleted groups treated with vehicle, cyclo (Leu-Gly) or MIF did not differ. A greater hypothermic response (1.5°C) to apomorphine was observed in rats withdrawn from morphine for 24 h. Rats pretreated with cyclo (Leu-Gly) or MIF and withdrawn from morphine showed a hypothermic response to apomorphine which was similar to that seen in rats implanted with placebo pellets (Table 5).

Discussion

The present findings suggest that MIF, a linear tripeptide and cyclo (Leu-Gly), an analogue derived from MIF, inhibit the development of tolerance to morphine in the rat. Not only did multiple injections of MIF and cyclo (Leu-Gly) inhibit the tolerance but the same effect was obtained by single injections, although much higher doses of the peptides were required. Multiple injections of the peptides in rats implanted with placebo pellets did not modify morphine-induced analgesia.

These data are consistent with previous results obtained in the rat (Bhargava, 1980b) where it was shown that daily administration of 2 mg/kg of cyclo (Leu-Gly) significantly inhibited tolerance to the analgesic effect of morphine. These data are also consistent with work in mice with MIF and cyclo (Leu-Gly) (Bhargava *et al.*, 1980) and with another analogue of MIF, Z-Pro-D-Leu (Walter *et al.*, 1978).

MIF is the hypothalamic factor which inhibits the release of melanocyte stimulating hormone from the anterior pituitary (Nair, Kastin & Schally, 1971). Oxytocin has been shown to serve as a precursor for MIF. This COOH-terminal tripeptide portion of oxytocin can be released enzymatically from the hormone by a membrane-bound hypothalamic enzyme (Walter, Griffiths & Hooper, 1973). Oxytocin and analogues like MIF and cyclo (Leu-Gly) were reported to facilitate the development of morphine tolerance and dependence in the rat (van Ree & de Wied, 1976). This effect of MIF was confirmed in mice (Szekeley, Miglecz, Kovacs, Tarnava, Ronai, Graf & Bajusz, 1979). However, studies with cyclo (Leu-Gly) (Bhargava, 1980b) and those of Schmidt *et al.* (1978) with oxytocin did not show facilitation of narcotic tolerance and the development of physical dependence.

Table 5 Effect of single injection of cyclo (Leu-Gly) and MIF on apomorphine-induced hypothermic response in placebo and morphine pellet-implanted rats

Treatment[a]	*Dose of peptide* (mg/kg s.c.)	*Body temperature, °C* (mean ± s.e.mean, $n = 6$) *Time after apomorphine (2 mg/kg i.p.) injection* (min) 0	20
Placebo			
Water	–	38.0 ± 0.2	36.9 ± 0.2
Cyclo (Leu-Gly)	4	37.9 ± 0.2	36.9 ± 0.2
MIF	8	37.9 ± 0.2	36.8 ± 0.2
Morphine			
Water	–	37.7 ± 0.2	36.2 ± 0.2*
Cyclo (Leu-Gly)	4	37.9 ± 0.2	36.9 ± 0.2
MIF	8	37.8 ± 0.2	36.9 ± 0.2

[a]Rats were injected with water, cyclo (Leu-Gly) or MIF 2 h before pellet implantation as described under Table 4. Twenty-four hours after pellet removal, rats were injected with apomorphine. The body temperature of each rat was measured at 0 and 20 min after apomorphine injection.
* $P<0.05$ vs. the reading at 20 min for all other groups.

The reason for the discrepancy between our results and those of van Ree & de Wied (1976) is not apparent at this time. However, the methodological differences between the two studies must be pointed out. In contrast to the study by van Ree & de Wied (1976) in which female rats were used, in the present study male rats were used to avoid the possible interfering variables induced by oestrous cycles of other pituitary and target-gland hormones. Secondly, the pellet implantation method for inducing morphine tolerance was used in this study whereas, van Ree & de Wied (1976) used multiple injection techniques.

Sex differences, thus, may be a factor for possible differences in the results. However, Lee & Ritzmann, (personal communication) have found that using exactly the same procedure of inducing morphine tolerance in both male and female rats, cyclo (Leu-Gly) failed to facilitate tolerance to the analgesic effect of morphine.

The precise mechanism by which MIF and cyclo (Leu-Gly) inhibit the development of narcotic tolerance is not known. In fact, the mechanism(s) by which narcotics produce tolerance and physical dependence is not yet delineated. One approach in recent years has been to study the effect of chronic administration of narcotics on various receptors in the brain. These studies have been summarized in a recent review by Overstreet & Yamamura (1979). Although there are many conflicting reports in the literature, it is believed that opiate receptors remain unchanged as tolerance develops (Dum, Blassig, Meyer & Hertz, 1979). Attempts have also been made to seek a possible relationship between opiate tolerance and changes in brain neurotransmitter receptors. Collier (1966) was the first to propose that on chronic administration of opiates, the postsynaptic receptors become supersensitive because of chronically decreased neurotransmission. Much of the work has been done with brain acetylcholine and dopamine receptors. Both behavioural and biochemical evidence suggest that supersensitivity of dopamine and acetylcholine receptors develops with chronic morphine treatment (Puri & Lal, 1973; Gianutsos, Hynes, Puri, Drawbaugh & Lal, 1974; Iwatsubo & Clouet, 1975). However, conflicting reports also exist, indicating lack of effect on dopamine stimulated adenylate cyclase (Kushinsky, 1975) and decreased dopamine receptor binding (Puri, Spaulding & Mantione, 1978) after chronic morphine treatment. Christie & Overstreet (1979), using [^{3}H]-spiroperidol and [^{3}H]-quinuclidinyl benzilate (QNB) as ligands for dopamine and acetylcholine receptors respectively, found that morphine-tolerant rats showed supersensitivity of dopamine receptors. However, in morphine withdrawn rats, the K_D for [^{3}H]-spiroperidol and B_{max} for [^{3}H]-QNB were decreased suggesting a subsensitivity of dopamine and acetylcholine receptors.

It has also been reported previously that morphine-tolerant mice (Ritzmann, Walter, Bhargava & Flexner, 1979) and rats (Bhargava, 1980b) exhibit supersensitivity to the dopamine agonist, apomorphine, and this can be blocked by treatment with cyclo (Leu-Gly). In the present study, an enhanced hypothermic response to apomorphine was observed in morphine-tolerant rats. This enhancement was

blocked by doses of MIF and cyclo (Leu-Gly) which inhibited the development of tolerance to morphine-induced analgesia. All these studies taken together strongly suggest a possible relationship between morphine tolerance and brain dopamine receptor sensitivity.

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