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Development of Narcotic Tolerance and Physical Dependence: Effects of Pro-Leu-Gly-NH_2 and cyclo (Leu-Gly)

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BHARGAVA, H. N., R. WALTER AND R. F. RITZMANN. *Development of narcotic tolerance and physical dependence: Effects of Pro-Leu-Gly-NH_2 and cyclo (Leu-Gly).* PHARMAC. BIOCHEM. BEHAV. 12(1) 73–77, 1980.— Administration of Pro-Leu-Gly-NH_2 (MIF) and cyclo (Leu-Gly) blocked the development of tolerance to and physical dependence on morphine, induced by the pellet implanation procedure in mice. Inhibition of tolerance development by peptides was evidenced by the presence of an analgesic response (increase in jump threshold) as determined by measuring the jump threshold to an increasing electric current, after a challenge dose of morphine (40 mg/kg). The same dose of morphine did not alter the jump threshold in morphine tolerant mice which were injected with saline prior to pellet implantation. The inhibition of the development of physical dependence on morphine by these peptides was evidenced by the antagonism of the hypothermic response which occurs during abrupt or naloxone-induced withdrawal. The naloxone-induced withdrawal jumping response was unaffected by these peptides. Dose-response experiments indicated that cyclo (leu-Gly) was much more potent than MIF in these tests. These peptides, when given after the development of tolerance and dependence, did not modify either the analgesic response to morphine or the symptoms of abrupt and naloxone-precipitated withdrawal. The inhibition of development of analgesic tolerance and physical dependence was not associated with changes in brain morphine concentration. The data indicate that these peptides do not interfere with the morphine-morphine receptor complex formation but alter a subsequent step in the genesis of some aspects of tolerance and dependence processes.

Morphine Analgesic tolerance Physical dependence Pro-Leu-Gly-NH_2 MIF cyclo(Leu-Gly) Naloxone Jump threshold Hypothermia

OXYTOCIN and its c-terminal fragment, Prolyl-leucyl-glycinamide (MIF) were shown to facilitate the development of physical dependence on morphine [10]. Substitution of an L-residue in the peptide hormone by the D-isomer in certain instances results in analogs with either decreased or no intrinsic activity but which retain the receptor affinity [6]. With this information, we recently reported that a dipeptide, benzyloxycarbonyl-Prolyl-D-Leucine (Z-Pro-D-Leu) is capable of inhibiting the development of tolerance to and physical dependence in mice [11]. These effects were achieved without affecting the acute responses of morphine, i.e., Z-Pro-D-Leu did not alter the analgesic response of mice in morphine naive animals. Furthermore, once the dependence had developed, Z-Pro-Leu did not modify the symptoms of morphine withdrawal. Subsequent structure-activity relationship studies utilizing the hypothermic response, which occurs during naloxone-induced withdrawal, as an index of the degree of physical dependence, revealed several structural analogs with greater potency than Z-Pro-D-Leu. The endogenous peptide MIF and the cyclic dipeptide, cyclo (Leu-Gly) appeared to be two potent peptides in this test system [12].

In the previous studies [11,12], peptides were administered daily, i.e., prior to and during the course of morphine pellet implantation. To determine the minimum dose necessary for the inhibition of morphine dependence, the present study utilized a single subcutaneous (SC) injection of the peptides given 2 hr prior to morphine treatment. A dose-response relationship has been determined. In the present series of experiments, the effect of these peptides on the development of physical dependence as measured by the intensity of abrupt and naloxone precipitated withdrawal responses, like body weight changes and stereotyped jumping behavior has been studied. The effect of these peptides on the development of tolerance to morphine has also been studied. Finally, the effects of peptide treatment on the brain morphine concentration in morphine tolerant-dependent mice have also been determined.

METHOD

Male Swiss Webster mice weighing 26 ± 4g (S.D.) (Scientific Small Animals, Inc., Arlington Heights, IL) were housed for at least four days prior to being used in a room

with controlled temperature (23 ± 1°C), humidity (65 ± 2%) and light (L 0600–1800 hr). The animals were given food and water ad lib.

Effect of MIF and cyclo (Leu-Gly) on the Development of Tolerance to Morphine

Mice were divided into three groups. One group of mice received SC injection of water (vehicle). The second and third group received SC injections MIF or cyclo (Leu-Gly) [8] dissolved in water at the appropriate dose. Two hours post injection mice were further subdivided; each subgroup was implanted subcutaneously with either a placebo or morphine (containing 75 mg free base) pellet [1]. The pellets were removed 72 hr after the implantation. The effect of peptide treatment on morphine analgesia and on the overt signs of physical dependence were also determined. In this case the peptides were given only on the third day of morphine pellet implantation, that is, 24 hr prior to pellet removal.

Tolerance to the analgesic effect of morphine was measured by injecting morphine sulfate (40 mg/kg) intraperitoneally (IP), 24 hr after the removal of placebo or morphine pellets. The analgesic response was determined by measuring the jump threshold to an increasing electric current on an electrified grid attached to a BRS/LVD shock generator/scrambler. The level of analgesia was determined by comparing the change in threshold just prior to, and 30 min. after the morphine injection. The statistical significance was determined by using the Students' *t*-test.

Effect of MIF and cyclo (Leu-Gly) on the Development of Physical Dependence on Morphine

Mice were treated with vehicle, MIF, cyclo (Leu-Gly) and implanted with pellets as described above. To determine the effect of peptide treatment on the development of physical dependence on morphine, the intensity of both abrupt and naloxone-precipitated abstinence syndrome was determined [1]. In abrupt withdrawal, the body temperature and body weight of each mouse in different treatment groups was determined over an 8 h period at various time intervals after pellet removal. The data are expressed as mean ± S.E.M. for 8 hr observation. An interrelationship of various symptoms of naloxone precipitated withdrawal in morphine dependent rodents has been demonstrated [2]. To precipitate withdrawal, naloxone, a narcotic antagonist was injected IP at a dose of 0.1 mg/kg, 1 hr after the pellet removal. The rectal temperature of each mouse was measured just prior to and at 30 min after the naloxone injection. The data were expressed as the difference between the two readings. The effect of each peptide treatment was determined by comparing the degree of change in rectal temperature in vehicle and peptide treated groups of mice.

The effect of peptide was also determined on the naloxone-induced withdrawal stereotyped jumping response. Six hours after the morphine pellet removal, mice were injected SC with a challenge dose of naloxone. Mice were placed on a circular platform immediately after naloxone injection and the number of mice jumping off the platform within a 15 min observation period was recorded. Three doses of naloxone, using 8 to 10 mice for each dose, were used to compute the naloxone ED_{50} in each group. The data were analyzed as described previously [1].

Effect of MIF and cyclo (Leu-Gly) on Brain Morphine Levels in Morphine-Dependent Mice

Brain levels of morphine were measured in morphine dependent mice that had received either vehicle or peptide injections. Mice were treated with peptides and implanted with morphine pellets as described under tolerance experiments. Mice from each treatment group were decapitated 72 hr after the morphine pellet implantation. Brains were rapidly removed, frozen on dry ice, and stored at −80°C until assayed for morphine. Morphine concentrations were determined fluorometrically as described before [3].

RESULTS

Effect of MIF and cyclo (Leu-Gly) on the Development of Tolerance to Morphine

Administration of both MIF and cyclo (Leu-Gly) before morphine pellet implantation inhibited the development of tolerance to morphine. In placebo pellet implanted mice, administration of morphine sulfate (40 mg/kg) increased the jump threshold significantly ($p<0.05$) (Table 1). The same dose of morphine, however, did not alter the jump threshold in morphine dependent mice which were injected with vehicle, indicating the presence of tolerance to morphine. Tolerance to morphine did not develop in mice which were injected with MIF or cyclo (Leu-Gly) prior to morphine pellet implantation as evidenced by an increase in the jump threshold following an injection of morphine (Table 1). When the peptides were given on day 3 of morphine pellet implantation, that is, after significant tolerance had already developed, these peptides did not block the development of tolerance to morphine.

Effect of MIF and cyclo (Leu-Gly on the Development of Physical Dependence on Morphine.

A single injection of 7.2 μmoles/kg of either MIF or cyclo (Leu-Gly) given prior to implantation of a morphine pellet prevented the development of physical dependence on morphine as evidenced by the prevention of hypothermia which occurs during abrupt withdrawal in morphine dependent rodents which are treated with vehicle. Eight hours after the pellet removal (abrupt withdrawal) the body temperature of mice injected with vehicle, MIF or cyclo (Leu-Gly) and implanted with placebo pellets did not differ (Table 2). The vehicle injected mice which were implanted with morphine pellets exhibited significant hypothermic response at 8 hours following the pellet removal as compared to vehicle injected placebo pellet implanted mice. The body temperature of mice which received peptide injections and morphine pellets was significantly higher than morphine treated mice which were injected with the vehicle (Table 2). A similar effect was found during naloxone-precipitated withdrawal; the peptide pretreatment prevented the development of physical dependence on morphine as measured by the hypothermic response (Table 3). A decrease of 1.31°C in body temperature was recorded following naloxone injection. MIF and cyclo (Leu-Gly) prevented this decrease in temperature. Dose-response analysis indicated that cyclo (Leu-Gly) was more potent than MIF. The lowest dose at which cyclo (Leu-Gly) was found to be active was 2.8 μmoles/mg, whereas, the minimum effective dose of MIF was 7.2 μmoles/kg. When injected on the third day of morphine pellet implantation,

TABLE 1

EFFECT OF PEPTIDES ON THE DEVELOPMENT OF TOLERANCE TO MORPHINE INDUCED ANALGESIA

Treatment*	Day of injection	Pellet	N	Jump threshold Meter units ± SEM Pre-injection	Post-injection
Saline	3	Morphine	20	4.07 ± 0.24	4.31 ± 0.33
MIF	3	Morphine	10	4.40 ± 0.60	5.00 ± 1.00
MIF	0	Morphine	30	4.70 ± 0.36	6.16 ± 0.41†
Cyclo(Leu-Gly)	3	Morphine	10	4.40 ± 0.40	4.80 ± 0.38
Cyclo(Leu-Gly)	0	Morphine	30	5.03 ± 0.32	6.59 ± 0.32†
Saline	3	Placebo	14	4.89 ± 0.29	6.15 ± 0.23†
MIF	0	Placebo	12	4.80 ± 0.14	5.80 ± 0.17†
Cyclo(Leu-Gly)	0	Placebo	10	4.60 ± 0.19	5.80 ± 0.23†

*Peptides were injected SC at a dose of 7.2 μmoles/kg. Mice were made morphine-dependent by subcutaneous implantation of 75 mg morphine pellets for a period of 3 days. At 24 hr after removal of the pellets, the animals were tested for jump threshold prior to and 30 min after an IP injection of morphine, 40 mg/kg.

†$p<0.05$ pre- vs. postinjection threshold.

TABLE 2

EFFECT OF PEPTIDES ON HYPTHERMIC RESPONSE DURING ABRUPT WITHDRAWAL IN MORPHINE DEPENDENT MICE

Treatment*	Pellet	N	Body temperature °C ± SEM
Vehicle	Placebo	14	36.73 ± 0.09
MIF	Placebo	12	36.63 ± 0.12
Cyclo(Leu-Gly)	Placebo	10	36.78 ± 0.11
Vehicle	Morphine	30	33.88 ± 0.18
MIF	Morphine	30	35.97 ± 0.24†
Cyclo(Leu-Gly)	Morphine	20	36.01 ± 0.32†

*Mice were injected with vehicle or 7.2 μmole/kg of peptide. Two hours later they were subdivided and implanted with either placebo or morphine pellets. The pellets were removed and the body temperature was determined at 8 hr after the pellet removal.

†$p<0.05$ vs. vehicle-morphine group.

TABLE 3

EFFECT OF PEPTIDES ON THE DEVELOPMENT OF PHYSICAL DEPENDENCE ON MORPHINE AS MEASURED BY NALOXONE-INDUCED HYPOTHERMIA IN MORPHINE DEPENDENT MICE

Treatment*	Dose μmole/kg	N	Body temperature Δ ± SEM
MIF	7.2	16	+0.25 ± 0.09†
MIF	5.6	13	−0.61 ± 0.32
MIF	4.0	8	−0.66 ± 0.32
MIF	2.8	8	−0.67 ± 0.19
MIF	1.6	10	−1.10 ± 0.53
MIF Day 3	7.2	10	−1.08 ± 0.51
Cyclo(Leu-Gly)	7.2	15	−0.02 ± 0.22†
Cyclo(Leu-Gly)	5.6	9	+0.62 ± 0.21†
Cyclo(Leu-Gly)	2.8	6	−0.16 ± 0.17†
Cyclo(Leu-Gly)	1.6	9	−1.73 ± 0.38
Cyclo(Leu-Gly)	0.16	8	−0.97 ± 0.12
Cyclo(Leu-Gly) Day 3	7.2	10	−1.26 ± 0.68
Vehicle	—	32	−1.31 ± 0.11

*Mice were made morphine-dependent by SC implantation of 75 mg morphine pellets for a period of 3 days. One hour after removal of pellets the aminals were injected with naloxone (0.1 mg/kg). Body temperature was measured just prior to and 30 minutes after the injection of naloxone and is expressed as their difference.

†$p<0.01$ vs. vehicle control.

these peptides did not alter the degree of hypothermia in morphine-dependent mice (Table 3).

Neither of the peptides at 7.2 μmole/kg had any observable effect on naloxone-induced stereotyped jumping behavior. The naloxone ED_{50} in the vehicle and peptide treated groups remained identical and was 20 μg/kg (data not shown). The peptides altered responses to chronic morphine treatment without altering brain morphine levels. Brain morphine levels in vehicle, MIF and cyclo (Leu-Gly) treated morphine dependent mice were identical and were 277, 292 and 265 ng/g, respectively.

DISCUSSION

The present studies indicate that a single subcutaneous injection of MIF or cyclo (Leu-Gly) given prior to chronic morphine treatment prevents the development of tolerance to and physical dependence on morphine. These peptides did not alter the tolerant or dependent state when they were injected after the development of the tolerance and dependence, i.e., the blockade of the development of tolerance to morphine by these peptides occurred without altering the analgesic response to morphine in naive mice or in mice in which tolerance had already developed. Similarly, the blockade of the development of hypothermic response during withdrawal in morphine dependent mice was not ob-

served if the peptides were given after the physical dependence had already occurred.

The development of tolerance to morphine was evidenced by a lack of analgesic response to morphine in the jump threshold test and the blockade of tolerance development by the peptides was evidenced by the return of the analgesia following morphine injection. Administration of morphine which increased the jump threshold in placebo pellet implanted mice failed to do so in morphine pellet implanted mice. Morphine dependent mice which were treated with the peptides prior to pellet implantation showed the analgesic response to morphine as evidenced by increases in jump threshold. These results are very similar to that obtained by Z-Pro-D-Leu in our previous study [11] which was given prior to and during the development of tolerance to morphine. Thus, a single injection of MIF and cyclo (Leu-Gly) were effective in blocking the development of tolerance to the analgesic effect of morphine.

One of the characteristic symptoms of morphine dependence is the hypothermic response observed during either abrupt or antagonist induced withdrawal. We have earlier shown that in the rat, the degree of dependence development can be corelated with the intensity of naloxone-induced withdrawal hypothermia [2]. Both MIF and cyclo (Leu-Gly) were effective in blocking this hypothermic response. Analysis of the dose-response relationships indicated that on a molar basis, cyclo (leu-Gly) was approximately two and a half times more potent than MIF. Both peptides, however, were unable to modify the stereotyped withdrawal jumping response under the present treatment conditions, since the naloxone ED_{50} values in vehicle and peptide treated morphine dependent mice were virtually identical.

Similar effect on naloxone-induced jumping response was seen with Z-Pro-D-Leu [11] and several other peptides [12]. The results with MIF are also in agreement with those reported recently by Szekeley *et al.* [9] which appeared while our manuscript was under review. Furthermore, our work with other peptides like, thyrotropin releasing hormone indicates that this drug given centrally antagonizes the jumping response as well as the hypothermia, however, when administered by peripheral route does not affect jumping response but antagonizes the development of withdrawal hypothermia [4]. It appears, therefore, that peripheral administration of behaviorally active peptides does not block the jumping response.

The inhibition of the development of morphine tolerance and physical dependence processes by MIF and cyclo (Leu-Gly) were achieved without altering the level of morphine in the brain at the time of pellet removal. Three days after the pellet implantation, the brain morphine concentration in vehicle or peptide treated mice did not differ. We have previously shown that agents which inhibit development of narcotic dependence do not alter brain morphine concentration [1].

van Ree and de Wied [10] have reported that neurohypophyseal peptides including oxytocin, MIF, and cyclo (Leu-Gly) facilitate the development of morphine dependence in the rat. The effect of MIF was confirmed by Szekely *et al.* [9]. However, Schmidt *et al.* [7] failed to show facilitation of narcotic tolerance development by vasopressin and oxytocin in mice. In the present study, we have shown that MIF and cyclo (Leu-Gly) both inhibit the development of tolerance and physical dependence on morphine. The disparity between the above studies may be due to species differences, routes of morphine administration or the doses used.

van Ree and de Wied [10] used female rats. By contrast, we used male mice in order to avoid the possible interfering variables induced by estrous cycles of other pituitary and target-gland hormones. Sex differences may be a possible reason for discrepancies among these studies. It has been suggested that the modification of development of tolerance and physical dependence by neurohypophyseal hormones and related peptides may be related to the effects of these peptides on learning [10], hence these peptides may facilitate acquisition of a learned component of responses to chronic exposure to morphine. In our previous study [11], Z-Pro-D-Leu inhibited the development of tolerance to and physical dependence on morphine without producing any effect on memory. Further, learning or memory as a component of altered physical dependence signs [10] seems unlikely since abstinence had not been induced previously in those animals.

The suggestion that these peptides could alter the rate of tolerance development without affecting tolerance end points [7] is possible but our studies and those of others [7] have found no other drug treatment which alters the rate of narcotic tolerance without modifying the maximum attainable tolerance.

Previous workers [9,10] have used multiple injection technique, while in our studies pellet implantation procedure was used to induce morphine tolerance and physical dependence. This difference, however, should not produce diagonally opposite results.

The mechanism(s) by which these peptides produce their inhibitory effect on morphine tolerance and physical dependence are not known at present. Although oxytocin, Z-MIF, Z-Pro-D-Leu and Leu-Gly NH_2 were found to affect presynaptic dopamine mechanisms in the extrapyramidal system in rat in the same direction [5], differential effects are produced by these drugs on morphine tolerance and physical dependence. Thus, oxytocin has been reported to either facilitate [10] or produce no change [7], while Z-Pro-D- Leu and Z-MIF inhibited [11] the development of tolerance and physical dependence process. Further studies are in progress to delineate the mechanisms by which these peptides modify responses to chronic narcotic administration.

In summary, the present studies clearly demonstrate that it is possible to inhibit the development of tolerance to morphine without affecting its analgesic response. Similarly, the physical dependence development process can be blocked. In the doses employed, so far we have not observed any overt toxicity of these peptides. The clinical applications of agents like MIF and cyclo (Leu-Gly) are obvious.

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