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Inhibition by Z-Pro-D-Leu of development of tolerance to and physical dependence on morphine in mice

(peptides/analgesia/amnesia/withdrawal)

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ABSTRACT **The peptide Z-Pro-D-Leu, injected daily in mice receiving morphine chronically, was found to prevent development of physical dependence as measured by changes in body temperature and body weight due either to abrupt or to naloxone-induced withdrawal. On the other hand, administration of Z-Pro-D-Leu only on the last day of morphine treatment did not alter the overt signs of withdrawal. Daily administration of Z-Pro-D-Leu was also effective in blocking the development of tolerance to the analgesic and the hypothermic effects of subsequent challenge doses of morphine. However, the peptide treatment did not alter the acute effects of a challenge dose of morphine on either analgesia or body temperature. No effects on memory were noted, as evaluated in a one-trial passive avoidance task. Clinical implications of the use of Z-Pro-D-Leu are discussed.**

Certain neurohypophyseal hormones, their analogs, and disulfide-containing cyclic fragments of these hormones facilitate development of physical dependence on and tolerance to actions of morphine (1–3). Several of these peptides, notably vasopressin and its analogs, can modify various aspects of behavior including acquisition and extinction of conditioned responses in lower species as well as of memory in man (4–10). In addition, non-disulfide-containing linear hormone fragments, such as Pro-Leu-Gly-NH_2 (melanotropin-release inhibiting factor) (11), Z-Pro-Leu-Gly-NH_2 (7), the enzymatically stable cyclo(Leu-Gly) (12), and Pro-Arg-Gly-NH_2 (13) also exhibit these effects (7, 8, 13).

Recently, in comparing the relative potencies of these peptides in facilitating the development of physical dependence on and tolerance to morphine, van Ree and de Wied (3) found not only that oxytocin and 8-arginine vasotocin are more effective than 8-arginine vasopressin but, moreover, that Pro-Leu-Gly-NH_2 and cyclo(Leu-Gly) are as effective as oxytocin in these tests.

Replacement of an L residue in a peptide hormone or agonistic analog by the D isomer has been reported to produce, in certain instances, a competitive inhibitor or partial agonist—findings that might be explained by steric misplacement of an "active element" (14) from its preferred orientation in the "active site" (15) such that intrinsic activity is decreased or even lost with retention of receptor affinity (14, 16).

On the basis of this background it was decided, as a first step in a series of studies, to evaluate the effect of Z-Pro-D-Leu on the development of physical dependence on and tolerance to morphine.

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MATERIALS AND METHODS

The Z-Pro-D-Leu (17) used was prepared in these laboratories (18). Male C57BL/6J, Swiss Webster, and ICR mice weighing 26 ± 4 g (±SD) were used in these studies.

Mice were randomly divided into two groups. One group received subcutaneous injections of water (vehicle); the other group received Z-Pro-D-Leu (50 μg per mouse on day 1). Two hours later, the mice were then further subdivided and each subgroup was implanted with placebo or morphine pellets. The injections of vehicle and Z-Pro-D-Leu were repeated 24 and 48 hr after the first injection in their respective groups. Morphine pellets, containing 75 mg of morphine (free base), were implanted subcutaneously between 1000 and 1100 and were removed 3 days later at the same time (19). The control animals were implanted with placebo pellets. Body temperature and weight were measured daily at 1100. Temperature was measured by using a lubricated rectal probe (inserted 2.5 cm into the rectum) and telethermometer (model 43TA, Yellow Springs Instrument Co., Yellow Springs, OH).

The level of analgesia was tested on each of the 3 days of the experiment. This was accomplished by measuring the jump threshold to an increasing electric current in an electrified grid attached to a BRS/LVE no. SGS-004 shock generator/scrambler (20). Mice were tested only once and thereafter discarded from the experiment. When determining the effect of Z-Pro-D-Leu on overt signs of physical dependence (body temperature and weight) (21) and tolerance (body temperature and analgesia) (20), the peptide or vehicle was injected only once on the third day of the morphine or placebo pellet implantation (24 hr prior to the removal of the pellet).

To determine the effects of peptide treatment on development of physical dependence, the abstinence syndrome was precipitated by using the morphine antagonist naloxone (Endo Laboratories Inc., NY) at the appropriate dose (0.1, 0.25, or 0.50 mg/kg) injected intraperitoneally 1 hr after removal of the morphine and placebo pellets. Pellet removal was performed 24 hr after the last peptide injection. These mice were monitored for changes in body temperature (see above) and body weight at 15-min intervals for 1 hr after the naloxone injection. Effects of removing the pellets (withdrawing morphine) were also assessed by utilizing body temperature and weight as physical dependence parameters; these measurements were made at 2-hr intervals for 10 hr after the removal of the pellets. Placebo-implanted mice were treated in the same manner as the morphine pellet-implanted mice (21).

Tolerance to hypothermic and analgesic effects of morphine injected intracerebroventricularly (ICV) was assessed 24 hr after the removal of the pellets in mice not injected with naloxone.

Abbreviation: ICV, intracerebroventricularly.

The ICV injection was 40 μg of morphine sulfate in 10 μl of solution (22). Body temperature was recorded prior to and at 10 and 15 min after injection of morphine. Analgesia was determined 1 hr after the ICV injection.

Brain levels of morphine were measured in mice that had received either the Z-Pro-D-Leu or vehicle injections. For these studies, on the third day after morphine implantation the mice were then sacrificed by decapitation, and the brains were rapidly removed, frozen on dry ice, and stored at −80° until assayed for morphine content. Brain morphine concentrations were determined fluorometrically (23).

Unless otherwise noted, statistical analysis for the above experiments were performed according to Winer (24) by the Student *t* test, and all data are expressed as means ± SD.

For behavioral studies using a one-trial passive avoidance task, male ICR, C57BL/6J, and Swiss Webster mice were used. The two-compartment passive avoidance box and details of the procedure have been described (25). Five seconds after entering the large compartment, ICR mice received 0.4 mA of scrambled foot shock for 0.8 sec; Swiss mice, 0.3 mA for 0.8 sec; and C57BL/6J mice, 0.2mA for 2 sec. Several agents have been found to be amnesic under these conditions. Immediately after training, the mice were injected subcutaneously with 100 μg of Z-Pro-D-Leu in saline or with saline alone. Mice were tested for retention 24 hr later. Mice that failed to enter the large compartment within 180 sec were removed and given a score of 180.

RESULTS

Effect of Multiple Injections of Z-Pro-D-Leu Prior to and during the Course of Pellet Implantation. On days 1 and 2 there was no significant difference in body temperature, in either Swiss Webster or C57BL/6J mice, between those receiving morphine and those receiving Z-Pro-D-Leu or vehicle; however, on the third day the morphine-treated Swiss Webster mice that had been injected with Z-Pro-D-Leu had a statistically significant ($P < 0.01$) lower body temperature (mean $\Delta = -1.5°$, $n = 14$) than the morphine-dependent mice injected with vehicle ($\Delta = -0.5°$, $n = 18$). Both groups displayed a biphasic response after morphine, with an initial hyperthermia on day 1 and a hypothermic response on subsequent days. Temperatures of placebo-implanted mice receiving either Z-Pro-D-Leu or vehicle did not change over the duration of pellet implantation. A similar relationship was found for the loss in body weight. Swiss Webster mice lost 16 or 14% in body weight when receiving Z-Pro-D-Leu or vehicle, respectively. The C57BL/6J mice given Z-Pro-D-Leu/morphine lost 14%; the vehicle/morphine group lost 11% in body weight during this period regardless of peptide or vehicle injections. There was one significant strain difference: a greater mortality during morphine treatment of C57BL/6J mice ($P = 0.06$, binomial test). However, there was no difference between strains in the number of deaths when the group given peptide/morphine was compared with the group given vehicle/morphine (C57BL/6J, $P = 0.32$; Swiss Webster, $P = 0.65$, binomial test). No significant differences were observed in brain morphine levels on the third day of morphine treatment between Swiss Webster mice injected with Z-Pro-D-Leu (288 ± 99 ng/g, $n = 6$) and vehicle-injected mice (310 ± 28 ng/g, $n = 6$).

Inhibition of Development of Tolerance to and Physical Dependence upon Morphine by Z-Pro-D-Leu. The jump threshold in vehicle-injected, morphine-dependent mice decreased, indicating development of tolerance to the analgesic properties of morphine. Threshold scores were 4.00 ± 0.18, 3.50 ± 0.52, and 2.55 ± 0.44, on days 1–3, respectively ($n = 6$ for each group). On the other hand, Z-Pro-D-Leu-injected mice receiving morphine displayed no attenuation of the analgesic effects (day 1 = 4.07 ± 0.27; day 2 = 4.27 ± 0.62; day 3 = 4.45 ± 0.64).

The effect of Z-Pro-D-Leu treatment on hypothermia and loss of body weight during precipitated abstinence is presented in Fig. 1. There was a significant ($P < 0.001$) difference between vehicle/morphine-treated and Z-Pro-D-Leu/morphine-treated mice at the time of maximum withdrawal, which occurred 8 hr after the removal of the pellets. There was no significant difference between the Z-Pro-D-Leu/morphine-treated mice and the two control groups for either of the variables at any of the time points.

Thirty minutes after injection of naloxone (0.1 mg/kg) in both strains of mice, as well as after doses of 0.25 or 0.50 mg/kg in the Swiss Webster mice, there was a significant hypothermia in vehicle/morphine-treated mice (Fig. 2). Naloxone injection into placebo-implanted mice that had received either peptide or vehicle was followed by a mild hyperthermic response. Those mice that had received the Z-Pro-D-Leu/morphine treatment responded in a manner similar to mice that had had no previous exposure to morphine, exhibiting a mild hyperthermia. On the other hand, mice that received the Z-Pro-D-Leu injection only on the third day responded as did the vehicle/morphine-treated mice—i.e., the injection of naloxone produced a hypothermic response in these animals that was not significantly different from that produced in the vehicle/morphine group.

Morphine-treated Swiss Webster mice given vehicle were tolerant to the hyperthermic ($P < 0.001$) and analgesic ($P < 0.001$) effects of the ICV injection of 40 μg of morphine compared to the vehicle placebo group (Table 1). This tolerance was evident for 24 hr after removal of the pellets. A similar diminished response after the ICV injection of 40 μg of morphine was found in C57BL/6J mice both for hypothermia ($P < 0.01$) and for analgesia ($P < 0.001$). Mice that had been given Z-Pro-D-Leu during chronic morphine treatment responded to the ICV injection of morphine in a manner that was not significantly different from that of morphine-naive mice but was significantly different from vehicle/morphine-treated mice ($P < 0.01$). When the peptide was given only on the third day of pellet implantation, the mice responded similarly to those that had received the vehicle injection and morphine—i.e., giving the peptide after the development of tolerance to morphine did not effectively alter the development of manifestation of tolerance. It is also significant that there was no difference in the response to the challenge dose of morphine between the placebo-implanted mice of either strain that had received the peptide and the placebo-implanted mice that received the vehicle injections ($P > 0.01$).

Effects of Treatment with Z-Pro-D-Leu on Memory. Effects of Z-Pro-D-Leu on memory in a one-trial passive avoidance task was tested in three strains of mice. For the ICR strain the median (±SEM) step-through latency value for peptide-treated mice was 180 ± 32.4 sec, $n = 10$; control 180 ± 29.6 sec, $n = 9$). The respective values for Swiss Webster and C57BL/6J mice were 180 ± 51.9 ($n = 4$; control 180 ± 17.5 sec, $n = 10$) and 180 ± 0.0 ($n = 4$; control 180 ± 0.0, $n = 6$), respectively, indicating that Z-Pro-D-Leu had no effect on retention of memory in any group.

DISCUSSION

It has been suggested that development of physical dependence upon certain central actions of morphine and other psychoactive drugs is a manifestation of central nervous system function analogous to memory or learning (26–28). Neurohypophyseal

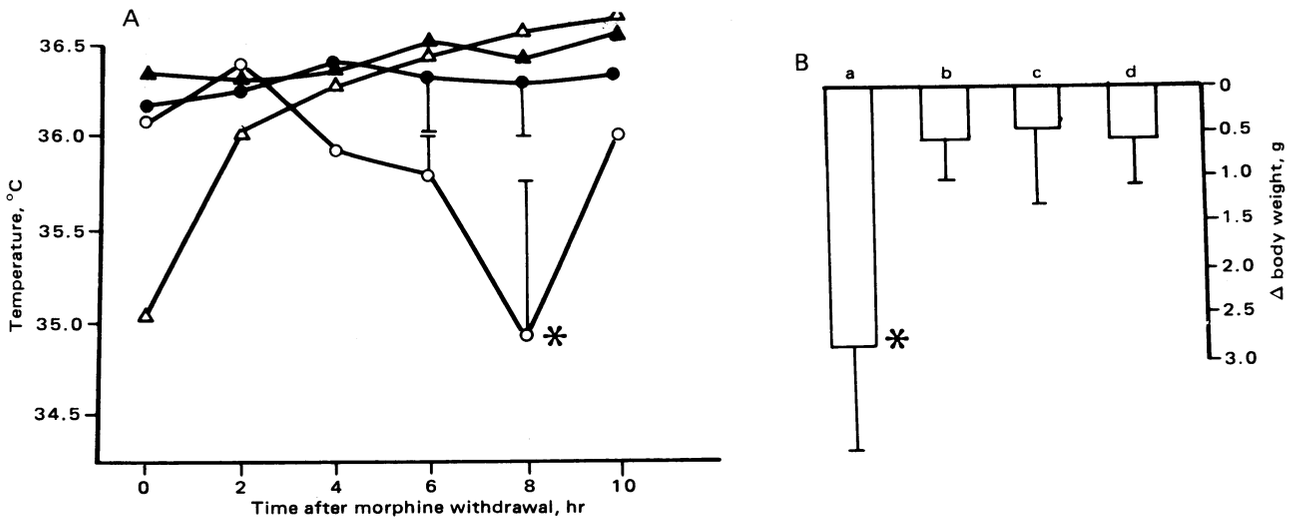

FIG. 1. Inhibition of development of physical dependence (abrupt withdrawal). Swiss Webster mice were implanted with either morphine or placebo pellets for a period of 3 days. After the removal of the pellets, body temperature and body weight were followed for 10 hr. (*A*) Change in body temperature. △, Z-Pro-D-Leu/morphine; ○, vehicle/morphine; ▲, Z-Pro-D-Leu/placebo; ●, vehicle/placebo. (*B*) Maximal loss in body weight during the same period. Bars: a, vehicle/morphine; b, Z-Pro-D-Leu/morphine; c, Z-Pro-D-Leu/placebo; d, vehicle/placebo. *, $P < 0.001$.

hormones as well as certain of their derivatives and fragments have been shown to alter memory and physical dependence upon and tolerance to morphine (1–10).

The present studies show that Z-Pro-D-Leu, conceptually derived from the central nervous system-active peptide family oxytocin, Pro-Leu-Gly-NH_2 or Z-Pro-Leu-Gly-NH_2 (7), is effective in blocking development of tolerance to actions of morphine on pain threshold, body temperature, and weight. The blockade appears to last in excess of 24 hr. Moreover, the dipeptide inhibits development of the physical dependence that accompanies chronic administration of morphine, because body weight and temperature of animals given both substances on a chronic basis did not change during abstinence precipitated by naloxone or by withdrawal of the morphine.

Although the data are clear, the mechanism by which Z-Pro-D-Leu interferes with development of tolerance and physical dependence is not certain. One might immediately suggest the possibility that the peptide antagonizes the opioid

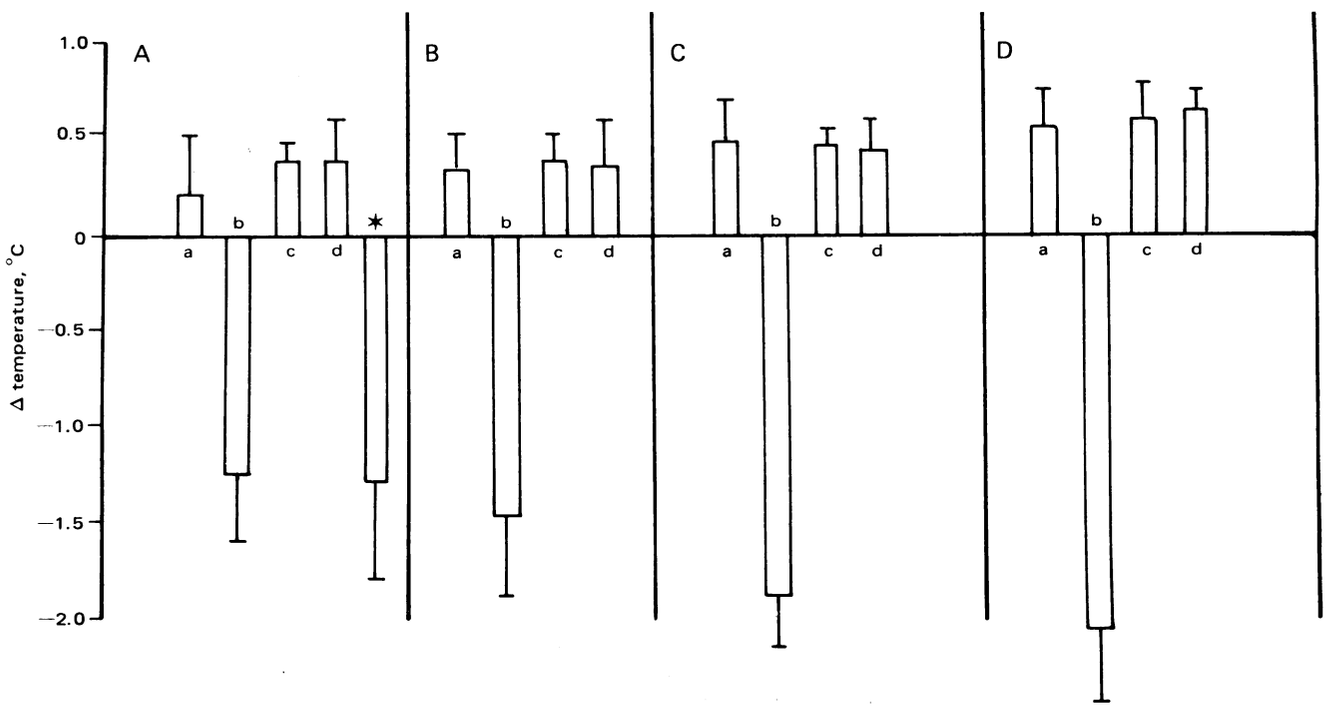

FIG. 2. Inhibition of development of physical dependence (naloxone-precipitated). The effect of increasing doses of naloxone on body temperature 30 min after injection in various groups of two strains of mice 1 hr after removal of the pellet. Bars: a, Z-Pro-D-Leu/morphine; b, vehicle/morphine; c, Z-Pro-D-Leu/placebo; d, vehicle/placebo; ★, the appropriate control groups (vehicle/morphine, vehicle/placebo, and peptide/placebo) for the single injection of Z-Pro-D-Leu given only on the third day of morphine treatment were tested (because these control groups did not differ from similar groups receiving multiple injections of peptide or vehicle they are not shown). Naloxone treatments: (*A*) C57BL/6J, 0.1 mg/kg; (*B*) Swiss Webster, 0.1 mg/kg; (*C*) Swiss Webster, 0.25 mg/kg; (*D*) Swiss Webster, 0.5 mg/kg.

Table 1. Inhibition of development of tolerance to morphine by Z-Pro-D-Leu in mice

Group	*n*	Δt, °C		Jump threshold
		At 10 min	At 15 min	
C57BL/6J:				
Vehicle/morphine	9	−0.73 ± 0.25*	−1.20 ± 0.47*	2.06 ± 0.36**
Z-Pro-D-Leu/morphine	9	−2.04 ± 0.42	−2.12 ± 0.52	3.82 ± 0.42
Z-Pro-D-Leu/morphine†	6	−0.75 ± 0.19*	−1.00 ± 0.16*	2.12 ± 0.34**
Vehicle/placebo	8	−1.81 ± 0.27	−1.94 ± 0.22	3.90 ± 0.68
Z-Pro-D-Leu/placebo	8	−1.76 ± 0.33	−2.19 ± 0.41	3.79 ± −.39
Z-Pro-D-Leu/placebo†	6	−1.85 ± 0.25	−1.88 ± 0.17	4.05 ± 0.85
Swiss Webster:				
Vehicle/morphine	8	−0.53 ± 0.17*	−0.58 ± 0.24*	2.65 ± 0.35**
Z-Pro-D-Leu/morphine	15	−1.10 ± 0.29	−1.32 ± 0.34	4.33 ± 0.54
Vehicle/placebo	8	−1.18 ± 0.28	−1.30 ± 0.48	4.25 ± 0.63
Z-Pro-D-Leu/placebo	8	−1.16 ± 0.30	−1.48 ± 0.46	4.18 ± 0.41

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 challenged with an ICV injection of 40 μg of morphine, and body temperature was measured 10 and 15 min after the morphine injection. At 1 hr after the administration of morphine, the jump threshold was determined. *n*, number of animals. Level of significance: *, $P < 0.01$; ** $P < 0.001$.
† Day 3 only.

so as, in effect, to decrease the dose. This seems not to be the case because the animals' weight, temperature, and response to noxious stimuli were the same in groups receiving peptide/placebo and vehicle/placebo. Furthermore, there appeared to be no difference between the behavior of the peptide/morphine and peptide/vehicle groups on day 1, indicating that, before the vehicle/morphine group became tolerant, the peptide neither enhanced nor antagonized the actions of morphine. It could be argued that Z-Pro-D-Leu somehow alters the metabolism of morphine, perhaps concurrently with altering sensitivity to the opioid. This seems unlikely because neither the agonistic actions of morphine nor its concentration in the brain was altered by the peptide. Finally, it seems unlikely that Z-Pro-D-Leu is "reversing" an action of morphine that results in the manifestation of tolerance and physical dependence because the phenomena were not reversed by the peptide once they had been established. These considerations would suggest Z-Pro-D-Leu does not act by directly or indirectly interfering with the primary morphine–receptor interaction but rather acts by altering or in essence preventing some subsequent step dependent upon this interaction.

The clinical implications of these results are obvious. Two of the most intriguing are (*i*) the possible use of a compound such as Z-Pro-D-Leu in patients treated with morphine for the control of chronic pain without developing addiction liability, and (*ii*) the possible use of this peptide, or similar ones, in treating a cycle of drug abuse. Moreover, it appears that Z-Pro-D-Leu has no undesirable effect on memory.

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