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Prolyl-leucyl-glycinamide, cyclo(leucylglycine), and derivatives block development of physical dependence on morphine in mice

(addiction/withdrawal/peptides/structure-activity relationship/antagonist)

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ABSTRACT **Pro-Leu-Gly-NH_2 (MIF) and several structural analogues, all injected in 50-μg doses daily in mice receiving morphine chronically, were found to prevent development of physical dependence as measured by changes in body temperature associated with naloxone-induced withdrawal. Dose–response studies, using again a protocol of daily injections of peptide at 50, 5, 0.5, 0.05, or 0.005 μg per mouse revealed MIF and cyclo(Leu-Gly) to be the most potent peptides and to be effective in blocking physical dependence to morphine at a dose as low as 0.5 and 0.05 μg per mouse, respectively. The benzyloxycarbonyl derivative of MIF, Pro-Leu, and Pro- -Leu exhibited significant activities down to a dose of 5 μg of peptide per mouse.**

Recent evidence (1) indicates that the dipeptide benzyloxycarbonyl-Pro-D-Leu is capable of blocking the development of tolerance to and physical dependence on morphine in mice; these effects were achieved without altering the analgesic potency of morphine.

In light of the potential clinical importance of these observations and their significance for the study of the mechanism of action of central nervous system-active peptides, it was decided to investigate whether more potent inhibitors could be found. The inhibitory properties of a selected number of peptides was evaluated over a broader dose range. Because, in our original studies, Z-Pro-D-Leu was equally effective in blocking tolerance, abrupt withdrawal, or naloxone-precipitated withdrawal (1), it was decided to use naloxone-precipitated withdrawal in mice as the bioassay in the current investigation for an evaluation of structure–function and dose–response relationships.

MATERIAL AND METHODS

A double-blind procedure was used for all experiments, and each peptide was tested in at least two independent experiments. Male Swiss Webster mice (Scientific Small Animal Farm, Inc., Melrose Park, IL) weighing 24 ± 2 g (mean ± SD) were used. The mice were housed five or six per cage in temperature (23 ± 1°C) and light (light 0600–1800 hr) controlled rooms and were kept in our laboratory for a minimum of 7 days prior to the initiation of experiments. Food (Purina Laboratory Chow) and water were available ad lib.

Mice were randomly divided into two groups. One group received subcutaneous injections of 0.1 ml of water (vehicle). The other group received peptide dissolved in 0.1 ml of water; in the case of the structure–activity studies, a single dose of 50 μg of peptide per mouse was given on day 1, and in the dose–response studies 50, 5, 0.5, or 0.005 μg of peptide was administered to the respective groups of mice. Two hours later the mice were implanted with morphine pellets. The injections of vehicle and respective peptides 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 hr and were removed 3 days later at the same time of day (2). Controls were as described (1); it had been determined (1) that the peptides alone had no effect on the response of the morphine-naive mice to naloxone.

To determine the effects of peptide treatment on development of physical dependence, the intensity of the antagonist-induced withdrawal syndrome was used as the criterion. Earlier studies from our laboratories demonstrated that the intensity of the body-weight loss, the hypothermia, and the stereotyped jumping during antagonist-induced withdrawal are related to the degree of dependence on morphine (3, 4). Thus, the greater the hypothermic response observed, the greater is the degree of dependence. The abstinence syndrome was precipitated by using the morphine antagonist naloxone (Endo Laboratories, Inc., Garden City, NY) at a dose of 0.1 mg/kg injected intraperitoneally 1 hr after removal of the morphine pellets. Pellet removal was performed 24 hr after the last injection of peptide or vehicle. These mice were monitored for changes in body temperature by using a lubricated rectal probe (inserted 2.5 cm into the rectum) and telethermometer (model 43TA, Yellow Springs Instrument); the first measurement was made just prior to naloxone administration and the rest were at 15, 30, and 60 min later. The results are expressed in the tables as the difference between the 0- and 30-min readings. At the time temperatures were being measured each mouse was rated as to the occurrence of additional withdrawal signs. A mouse was scored as withdrawing if at least two of the following symptoms were observed: shakes, jumping, and diarrhea.

RESULTS AND DISCUSSION

The structure–function analysis revealed that several peptides were effective in blocking the development of physical dependence on morphine (Table 1).

The naturally occurring peptide Pro-Leu-Gly-NH_2 (MIF; also known as MSH-release-inhibiting factor, MSH-R-IF) (5, 6) was found to be very effective when injected daily at a dose of 50 μg per mouse. The addition of a *N*-benzyloxycarbonyl (Z) group apparently did not alter the activity of the peptide in this testing situation, but addition of Z-Gly or substitution of pyro-Glu (□Glu) for the NH_2-terminal proline gave derivatives with reduced activity. Replacement of the proline residue by 3,4-dehydroproline (Δ^3Pro), deletion of the proline moiety,

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Abbreviations: Nomenclature is in accord with the IUPAC–IUB Rules on Biochemical Nomenclature [(1967) *J. Biol. Chem.* **242**, 555–557; and (1972) *J. Biol. Chem.* **247**, 977–983]. All optically active amino acids are of L configuration unless otherwise noted. MIF, Pro-Leu-Gly-NH_2; Z-MIF, benzyloxycarbonyl-MIF; Z, *N*-benzyloxycarbonyl group.

Table 1. Structure–activity relationship for Pro-Leu-Gly-NH_2 (MIF) in blocking naloxone-precipitated withdrawal in mice

Treatment	*n*	Δt,* °C	*P*†	Withdrawal, % of control‡
Pro-Leu-Gly-NH_2 (MIF)	8	+0.25 ± 0.24	0.001	0
Δ^3-Pro-Leu-Gly-NH_2	6	−1.53 ± 0.81	NS	100
Z-Pro-Leu-Gly-NH_2 (Z-MIF)	6	+0.07 ± 0.08	0.001	0
Z-Pro-Leu-Gly-$N(CH_3)_2$	6	−0.47 ± 0.15	NS	100
Z-Pro-Leu-Gly-COOH	5	−1.20 ± 0.39	NS	100
Z-Gly-Pro-Leu-Gly-COOH	9	−0.07 ± 0.72	0.05	22
□Glu-Leu-Gly-NH_2	9	−0.27 ± 0.72	0.05	44
Z-Leu-Gly-NH_2	13	−0.75 ± 0.72	NS	70
Pro-Leu	8	−0.13 ± 0.74	0.05	25
Z-Pro-Leu-NH_2	5	−0.95 ± 0.21	NS	100
Z-Pro-Leu	9	+0.08 ± 0.93	0.05	22
Z-D-Pro-D-Leu	6	+0.10 ± 0.56	0.01	0
Z-D-Pro-Leu	7	+0.17 ± 0.71	0.01	14
Z-Pro-D-Leu	11	−0.09 ± 0.58	0.01	9
Z-Pro-Gly	13	−0.79 ± 0.78	NS	69
Z-Pro-Ala	6	−0.74 ± 0.94	NS	63
Z-Pro-D-Ala-DCHA	9	−0.55 ± 0.90	NS	50
Z-Pro-Ile	9	−1.19 ± 0.91	NS	78
Z-Pro-Val	6	−0.44 ± 0.48	NS	63
Z-Pro-Glu	11	−0.63 ± 0.88	NS	45
Z-Pro-Gln	5	+0.08 ± 0.33	0.01	0
Z-Pro-Ser	13	−0.29 ± 0.73	0.05	39
Z-Pro-Met	6	+0.17 ± 0.23	0.001	0
Z-Pro-Phe	9	−1.03 ± 0.55	NS	89
Z-Pro-ΔPhe	9	−0.36 ± 0.58	0.05	22
Z-Pro-Tyr	5	+0.13 ± 0.31	0.01	0
Z-Ala-Pro	4	−1.20 ± 0.21	NS	100
Cyclo(Leu-Gly)	14	−0.02 ± 0.83	0.01	21
Cyclo(Leu-Ala)	5	−1.80 ± 0.96	NS	100
Cyclo(Pro-Phe)	5	−0.13 ± 0.55	0.05	20
Cyclo(Pro-D-Leu)	10	−0.24 ± 1.06	NS	40
Vehicle (control)	33	−1.18 ± 0.43	—	99

DCHA, dicyclohexylamine.

* Difference in body temperatures of the mouse determined just prior to naloxone injection (0.1 mg/kg) and 30 min thereafter; values are expressed as mean ± SD.

† Means compared by Student's *t* test. $P > 0.05$ was considered to be not significant (NS).

‡ Based on jumping, shakes, and diarrhea (animals showed two symptoms).

dimethylation of the primary carboxamide group, or replacement of the glycinamide moiety by glycine resulted in inactive derivatives of MIF. However, the free dipeptide Pro-Leu exhibited activity; and, as in our previous study (1), Z-Pro-D-Leu was active under the present test conditions as well. Reminiscent of another investigation (7) of ours, in which we found the COOH-terminal dipeptide of oxytocin, Leu-Gly-NH_2, and its optical isomer, D-Leu-Gly-NH_2, to be very effective in attenuating puromycin-induced amnesia in mice is the finding that all of the four possible optical isomers—i.e., Z-Pro-Leu, Z-D-Pro-Leu, Z-Pro-D-Leu, and Z-D-Pro-D-Leu—were able to block physical dependence on morphine. Likewise, the substitution of Gln, Met, or Tyr for Leu in Z-Pro-Leu gave potent derivatives, but the substitution by either Ser or ΔPhe gave peptides of reduced activity. All the above peptides may be considered to be analogues of MIF.

Another group of peptides that can yield active derivatives and that merit further study are the cyclic dipeptides. Among the few tested, cyclo(Leu-Gly) and cyclo(Pro-Phe) showed activity. This group of cyclic dipeptides is of particular interest because a substantial amount of research with cyclo(Leu-Gly) has revealed it not only to be active behaviorally (7–9) and to be able to cross the blood–brain barrier (10) as well as intestinal tissue (11) intact but also to be stable to enzymatic degradation in brain for at least 96 hr (12).

Dose–response experiments revealed MIF and cyclo(Leu-Gly) to be the most potent peptides tested to date in blocking physical dependence to morphine (Table 2). MIF retained its effectiveness until doses of less than 0.5 μg per mouse were used; doses of either 0.05 or 0.005 μg per mouse failed to produce any alteration in the withdrawal response. Cyclo(Leu-Gly) was still effective in blocking physical dependence at a dose as low as 0.05 μg per mouse. Z-MIF, Z-Pro-Leu, and Z-Pro-D-Leu exhibited significant activities until a dose of less than 5 μg per mouse was administered.

It has been reported by van Ree and de Wied (13) that neurohypophyseal peptides including oxytocin, MIF, and cyclo(Leu-Gly) facilitate the development of morphine dependence in rats. Although we obtained similar results in mice in the case of oxytocin (unpublished data) with the method of morphine implantation as described (1, 2), the current results with MIF and cyclo(Leu-Gly) in mice are apparently in disagreement with those reported by van Ree and de Wied (13) for these compounds in rats. The disparity between the two studies may be due to differences of species, routes of morphine administration, or doses used.

Little can be said at present with respect to mechanism of action of these peptides on blocking physical dependence on morphine, although evidence is accumulating to suggest that dopaminergic systems may not play a major role. Oxytocin,

Table 2. Dose–response effects of MIF and derivatives on naloxone-precipitated withdrawal

Treatment	Dose, μg	*n*	Δt,* °C	*P*†	Withdrawal, % of control‡
MIF	50	8	+0.25 ± 0.24	0.001	0
	5	4	+0.08 ± 0.28	0.001	0
	0.5	5	+0.18 ± 0.39	0.001	0
	0.05	4	−1.50 ± 0.14	NS	100
	0.005	4	−1.05 ± 0.17	NS	100
Z-MIF	50	6	+0.07 ± 0.08	0.001	0
	5	6	−0.37 ± 0.26	0.05	33
	0.5	6	−0.65 ± 0.85	NS	66
Z-Pro-Leu	50	9	+0.08 ± 0.93	0.01	18
	5	4	+0.33 ± 0.26	0.01	0
	0.5	6	−1.17 ± 0.17	NS	100
Z-Pro-D-Leu	50	11	−0.09 ± 0.58	0.001	9
	5	4	−0.05 ± 0.37	0.01	25
	0.5	6	−1.47 ± 0.12	NS	100
Cyclo(Leu-Gly)	50	15	−0.19 ± 0.58	0.001	18
	5	5	+0.47 ± 0.32	0.001	0
	0.5	10	+0.30 ± 0.29	0.001	0
	0.05	10	+0.27 ± 0.70	0.01	20
Z-Pro-Phe	50	9	−1.03 ± 0.55	NS	99
	5	5	−0.69 ± 0.24	NS	100
	0.5	6	−0.69 ± 0.22	NS	100
	0.05	4	−1.60 ± 0.69	NS	100
Vehicle	0	33	−1.18 ± 0.43	—	100

For footnotes, see Table 1.

Z-MIF, Z-Pro-D-Leu, and Leu-Gly-NH_2 all were found to affect presynaptic dopamine mechanisms in the extrapyramidal system in the rat in the same manner and to a similar degree under the conditions tested (14). However, whereas oxytocin was found to facilitate the development of morphine tolerance and physical dependence in rats (13) and mice (unpublished data), Z-Pro-D-Leu (1) and Z-MIF inhibited formation of tolerance to or blocked development of physical dependence in mice. On the other hand, Leu-Gly-NH_2 had no effect on the development of physical dependence. These data would argue against the concept that these peptides alter the development of physical dependence on morphine through dopamine-mediated neurons. Currently, therefore, we see no apparent correlation between the effects of these peptides on morphine addiction and dopaminergic mechanisms in the extrapyramidal system. These conclusions are in line with our contention that dopamine is involved in the expression of symptoms of physical dependence but not in the development of the physical dependence process (15, 16).

In summary, in our hands MIF and cyclo(Leu-Gly) are the most potent peptides to date in blocking physical dependence on morphine in mice, and several additional structural analogues of MIF were found to exhibit a substantial degree of potency.

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1. Walter, R., Ritzmann, R. F., Bhargava, H. N., Rainbow, T. C., Flexner, L. B. & Krivoy, W. A. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4573–4576.
2. Bhargava, H. N. (1978) *Pharmacol. Biochem. Behav.* **8**, 7–11.
3. Bhargava, H. N. (1977) *Psychopharmacol.* **52**, 55–62.
4. Bhargava, H. N. & Matwyshyn, G. A. (1977) *Eur. J. Pharmacol.* **44**, 25–33.
5. Celis, M. E., Taleisnik, S. & Walter, R. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 1428–1433.
6. Nair, R. M. G., Kastin, A. J. & Schally, A. V. (1971) *Biochem. Biophys. Res. Commun.* **43**, 1376–1381.
7. Walter, R., Hoffman, P. L., Flexner, J. B. & Flexner, L. B. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 4180–4184.
8. Flexner, J. B., Flexner, L. B., Hoffman, P. L. & Walter, R. (1977) *Brain Res.* **134**, 139–144.
9. Flexner, J. B., Flexner, L. B., Walter, R. & Hoffman, P. L. (1978) *Pharmacol. Biochem. Behav.* **8**, 93–95.
10. Hoffman, P. L., Walter, R. & Bulât, M. (1977) *Brain Res.* **122**, 87–94.
11. Gallo-Torres, H. E. & Walter, R. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 509.
12. Rainbow, T. C., Flexner, J. A., Flexner, L. B., Hoffman, P. L. & Walter, R. (1978) *Pharmacol. Biochem. Behav.*, in press.
13. van Ree, J. M. & de Wied, D. (1976) *Life Sci.* **19**, 1331–1340.
14. Diamond, B. I., Walter, R., Sudakoff, G. S., Havdala, H. S., Oltmans, G. A. & Borison, R. L. (1978) in *Catecholamines: Basic and Clinical Frontiers*, ed. Usdin, E. (Pergamon, New York), in press.
15. Bhargava, H. N., Afifi, A. H. & Way, E. L. (1973) *Biochem. Pharmacol.* **22**, 2769–2772.
16. Bhargava, H. N. & Way, E. L. (1975) *J. Pharmacol. Exp. Ther.* **190**, 165–175.