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Physical Dependence of a Dermorphin Tetrapeptide Analog, [D-Arg^2, Sar^4]-Dermorphin (1–4) in the Rat

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NAKATA, N., S. SAKURADA, T. SAKURADA, K. KISARA, Y. SASAKI AND K. SUZUKI. *Physical dependence of a dermorphin tetrapeptide analog, [D-Arg^2, Sar^4]-dermorphin (1–4) in the rat.* PHARMACOL BIOCHEM BEHAV **24**(1) 27–31, 1986.—The characteristics of an analog of tetrapeptide dermorphin (H-Tyr-D-Arg-Phe-Sar-OH), [D-Arg^2, Sar^4]-dermorphin (1–4) were examined in comparison with morphine by the appearance of typical withdrawal signs upon cessation of administration or treatment with naloxone, an opioid antagonist. The dose of [D-Arg^2, Sar^4]-dermorphin (1–4) or morphine in the physical dependence test can be quantified by determining the ED_{50} to inhibit the tail-flick response to thermal stimuli. Doses from 8 to 64 times the ED_{50} doses were employed in the subcutaneous injection schedules. The cessation of [D-Arg^2, Sar^4]-dermorphin (1–4) or naloxone treatment was largely without effect on body weight, in contrast to a marked loss of weight in morphine-dependent rats. The tetrapeptide failed to substitute for morphine in morphine-dependent rats. The physical dependence of [D-Arg^2, Sar^4]-dermorphin (1–4) was revealed by the behavioral signs of withdrawal precipitated by naloxone. However, the scores of lacrimation, diarrhea and urination were much lower in chronically tetrapeptide-treated rats than in morphine-treated rats, though the score of teeth chatter was higher. These findings indicate that [D-Arg^2, Sar^4]-dermorphin (1–4) may differ from morphine in physical dependence.

Morphine [D-Arg^2, Sar^4]-dermorphin (1–4) Tail-flick test Dependence Withdrawal signs Rats

DERMORPHIN was first isolated from Amphibian skin [5] and found to produce a potent and long-lasting antinociceptive activity. The antinociceptive effect of this peptide is approximately 11 times more potent than that of morphine using the hot-plate test in mice and this effect was completely abolished by pretreatment with naloxone [2]. Broccardo *et al.* [2] suggested that both tolerance and physical dependence were consistently less marked with dermorphin than with morphine. In addition, dermorphin has been shown to possess a potent activity to inhibit the electrically induced contractions of the guinea pig ileum (GPI) and mouse vas deferens (MVD). The sample of dermorphin obtained through their synthesis displays typical opioid activity on GPI and is 50 times more potent than that of [Leu]enkephalin (Leu-ENK). In the MVD, dermorphin was approximately equipotent to Leu-ENK [14]. It was also proposed that the N-terminal tetrapeptide H-Tyr-D-Ala-Phe-Gly-NH_2 is the minimal segment required for the activity [15]. We have studied the structure-activity relationship of dermorphin [16,17] and found a systemically potent antinociceptive tetrapeptide (H-Tyr-D-Arg-Phe-Sar-OH), [D-Arg^2, Sar^4]-dermorphin (1–4).

We now report the characteristics of the physical dependence of [D-Arg^2, Sar^4]-dermorphin (1–4) in comparison with that of morphine using the withdrawal, substitution and naloxone-challenge tests in the rat.

METHOD

Male Wistar strain rats, weighing between 150–180 g, were used in the present experiment. The animals were housed at 22±2°C, food and water available ad lib. A standard light-dark cycle was maintained with a time-regulated light period from 9:00 a.m. to 9:00 p.m.

Morphine hydrochloride (Sankyo) and naloxone hydrochloride (Endo laboratories) were used. [D-Arg^2, Sar^4]-dermorphin (1–4) was synthesized by Sasaki *et al.* [16]. [D-Arg^2, Sar^4]-dermorphin (1–4) used in the present experiment showed a single spot on thin layer chromatography when 50 μg was applied to silica gel plates: Rf 0.30 in n-BuOH-acetic acid-water (4:1:5, upper phase), Rf 0.59 in n-BuOH-acetic acid-pyridine-water (15:10:3:12). All drugs were dissolved in saline and injected subcutaneously (SC).

The tail-flick test was adapted from the classical design of D'Amour and Smith [3]. The latency of the tail-flick response to heat focused on the tip of the tail was used as an index of antinociceptive effect and measured with a tail-flick unit (UGO BASIL, model-DS20) as previously described

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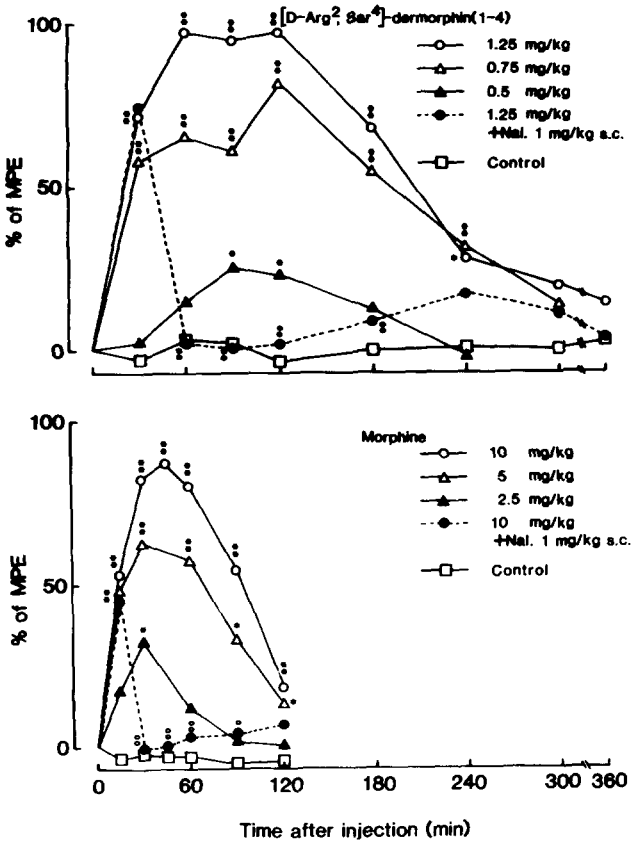

FIG. 1. Antinociceptive effects of different doses of [D-Arg², Sar⁴]-dermorphin (1–4) and morphine given by subcutaneous injection, and antagonism by naloxone as measured by the tail-flick test in rats. The ED_{50} of [D-Arg², Sar⁴]-dermorphin (1–4) was 0.605 (0.462–0.793) mg/kg, that of morphine 3.8 (2.1–7.0) mg/kg. Each point is the mean value obtained from 6–8 rats. Values which are significantly different from the saline-treated groups are indicated with $*(p<0.05)$, $**(p<0.01)$.

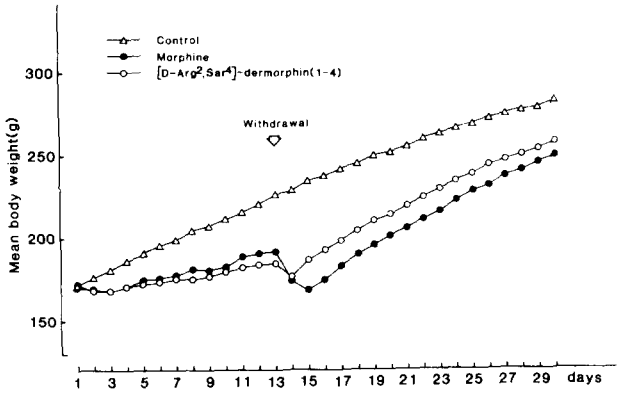

FIG. 2. Changes in body weight after abrupt cessation in rats chronically administered [D-Arg², Sar⁴]-dermorphin (1–4) and morphine during measurement. Abrupt withdrawal of the tetrapeptide and morphine was performed on day 13. Saline-treated animals served as controls. Each point is the mean value obtained from 10–11 rats. See the Method section for injection schedule.

[10]. The tail-flick latencies to thermal stimuli were determined at 15, 30, 45, 60, 90, 120, 180, 240, 300 and 360 min after [D-Arg², Sar⁴]-dermorphin (1–4) or morphine. Naloxone was given SC 15 min and 45 min after the administration of morphine and [D-Arg², Sar⁴]-dermorphin (1–4), respectively. A cutoff of 15 sec was imposed on animals failing to remove their tails from the light beam to avoid tail tissue damage. The antinociceptive effect for each rat was calculated according to the following formula and represented as % of maximum possible effect (MPE): % of MPE = $[(T_2-T_1)/(15-T_1)] \times 100$ where T_1 is a control latency obtained from the mean of two latencies measured before drug injection and T_2 was then measured for each animal at various intervals after drug injection. The mean control latency before drug injection was 5.37±0.14 sec. The median antinociceptive dose (ED_{50}) and their 95% confidence limits were determined by the method of Litchfield and Wilcoxon [11]. The ED_{50} values for morphine and [D-Arg², Sar⁴]-dermorphin (1–4) were calculated from the values obtained at 30–45 min and 90–120 min, respectively, which are the time of peak effect.

The withdrawal study was modified from the original method [18]. Morphine at 30 mg/kg/day equal to 8 times the ED_{50} for antinociceptive activity was administered SC twice daily (9:00 a.m. and 9:00 p.m.) for the first three days. Subsequently, 60 mg/kg/day on days 4, 5 and 6, 120 mg/kg/day on days 7, 8 and 9, 240 mg/kg/day on days 10, 11 and 12 were administered SC twice daily. On day 13, the effect of abrupt withdrawal of morphine on body weight and water and food consumption was tested by giving saline. The administration schedule of [D-Arg², Sar⁴]-dermorphin (1–4) was the same as that of morphine; the dose of the tetrapeptide increased 8 to 64 times the ED_{50}. The initial dose (4.8 mg/kg/day) of [D-Arg², Sar⁴]-dermorphin (1–4) was administered SC twice daily for the first three days. Subsequently, 9.6 mg/kg/day on days 4, 5 and 6, 19.2 mg/kg/day on days 7, 8 and 9, 38.4 mg/kg/day on days 10, 11 and 12 were administered SC twice daily. Control rats were treated with saline.

In the substitution study, repeated injections of morphine were given to rats according to the same schedule as used in the withdrawal study. Chronically morphine-treated rats were divided into two groups—12 animals received 38.4 mg/kg/day of [D-Arg², Sar⁴]-dermorphin (1–4) and 12 animals received 240 mg/kg/day of morphine on day 13, at end of treatment. On day 14, the effect of abrupt withdrawal was examined.

In the naloxone challenge study, the rats were given morphine or [D-Arg², Sar⁴]-dermorphin (1–4) SC for 9 days, according to the first 9 day schedule used in the withdrawal study. The last dose of agonists was given on day 9 at 9:00 p.m. At 9:00 a.m. on day 10, the rats were challenged with naloxone at a dose of 1 mg/kg, IP. Withdrawal severity was assessed for 15 min following the injection of naloxone. The following signs were scored by the method of Frederickson and Smits [6]: salivation, rhinorrhea, lacrimation, urination, diarrhea, erection, ejaculation, ptosis, teeth chatter, swallowing, tremor, hunchback posture, piloerection, irritability to handling, reaction to poking sharp object, escape behavior (jumping or digging), wet dog shakes, head shakes, foreleg shakes and writhing.

These data obtained were statistically analysed by an analysis of variance (ANOVA) [8] followed by either the Tukey's test or the Dunnett's test. A p value of less than 0.05 was taken as the level of statistical significance.

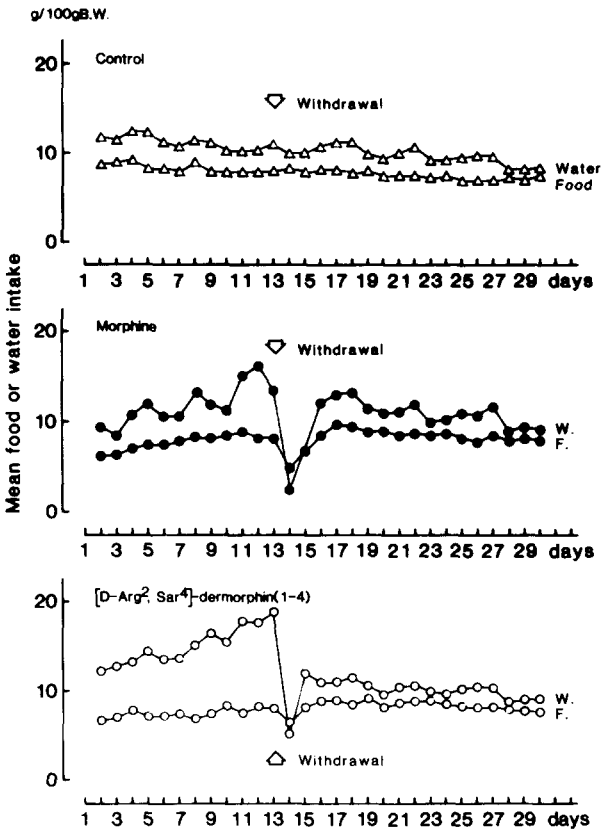

FIG. 3. Changes in the amount of food and water intake after abrupt cessation in rats chronically administered [D-Arg^2, Sar^4]-dermorphin (1–4) and morphine. Abrupt withdrawal of the peptide and morphine was performed on day 13. Saline-treated animals served as controls. Each point is the mean value obtained from 10–11 rats. See the Method section for injection schedule.

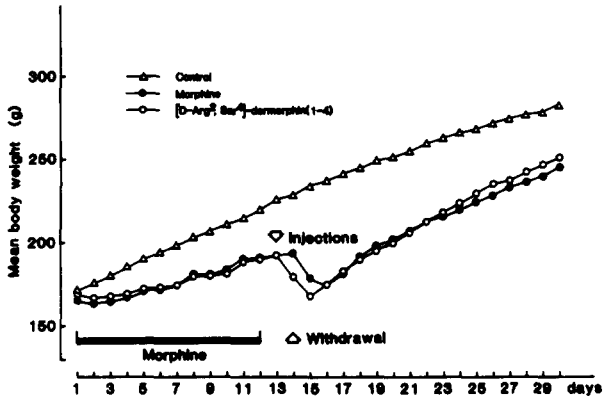

FIG. 4. Changes in body weight after injection of [D-Arg^2, Sar^4]-dermorphin (1–4) in rats chronically administered morphine. See the Method section for injection schedule. The rats were made dependent on morphine by a series of SC injections of morphine administered twice daily. The chronic morphine-treated rats were divided into two groups. One group received [D-Arg^2, Sar^4]-dermorphin (1–4), the other morphine on day 13, at the end of treatment. Both groups received no treatment after day 13. Saline-treated animals served as controls. Each point is the mean value obtained from 6 rats.

RESULTS

Analgesia as Measured by the Tail-Flick Method

[D-Arg^2, Sar^4]-dermorphin (1–4) and morphine administered SC were found to have dose-dependent antinociceptive activities in the rat tail-flick test (Fig. 1). The antinociceptive effect of [D-Arg^2, Sar^4]-dermorphin (1–4) peaked at 90–120 min and lasted from 240–360 min. In contrast, the peak time effect of morphine was seen at 45 min post-injection and the effect was completely absent at 180 min. Saline control did not induce significant effects. In the tail-flick test, the ED_{50} values were 0.605 (0.462–0.793) mg/kg for [D-Arg^2, Sar^4]-dermorphin (1–4) and 3.8 (2.1–7.0) mg/kg for morphine. From the calculation of ED_{50} values, the antinociceptive activity of [D-Arg^2, Sar^4]-dermorphin (1–4) was about 13 times more potent than that of morphine on a molar basis. [D-Arg^2, Sar^4]-dermorphin (1–4)-induced antinociception was markedly antagonized by pretreatment with naloxone (1 mg/kg, SC). The naloxone antagonizing effect on morphine-induced antinociception was almost the same as the effect on [D-Arg^2, Sar^4]-dermorphin (1–4)-induced antinociception.

Withdrawal Effects on Body Weight and Food and Water Intake

Figure 2 shows the effect of repeated SC administration of [D-Arg^2, Sar^4]-dermorphin (1–4), morphine and saline for 12 days. Abrupt withdrawal of [D-Arg^2, Sar^4]-dermorphin (1–4) on day 13 produced only a slight loss of body weight (approximately 4%) at 24 hours post-withdrawal as compared to the weight on the last day of this peptide treatment. In contrast, withdrawal of morphine on day 13 resulted in a sharp loss of body weight (approximately 12% at maximum) at 72 hours post-withdrawal. As indicated above, the morphine treated group exhibited a much more profound weight loss than the [D-Arg^2, Sar^4]-dermorphin (1–4)-treated group.Body weight loss after withdrawal of [D-Arg^2, Sar^4]-dermorphin (1–4), morphine and saline were not statistically significant during 12 hours (data not shown).

As shown in Fig. 3, the consumption of water intake was markedly depressed by withdrawal of [D-Arg^2, Sar^4]-dermorphin (1–4) and morphine at 24 hours. Only a slight depressed effect on food intake was seen in both groups.

Substitution Effects on Body Weight and Food and Water Intake

In the morphine-dependent rats, administration of [D-Arg^2, Sar^4]-dermorphin (1–4) could not substitute for morphine in the measurement of body weight and food and water consumption. The peptide had the same effect as morphine in food and water consumption in chronically morphine treated rats (Fig. 5). However, the body weight in the morphine-dependent rats injected with the peptide decreased 7% (13 g) on day 14 as compared with the value on day 13, whereas the administration of morphine maintained their weight on day 14 (Fig. 4).

Withdrawal Reaction Precipitated by Naloxone

The withdrawal reaction precipitated by naloxone (1 mg/kg, IP) after chronic treatment with [D-Arg^2, Sar^4]-dermorphin (1–4) and morphine was characterized by shaking including wet dog shakes, head shakes and foreleg

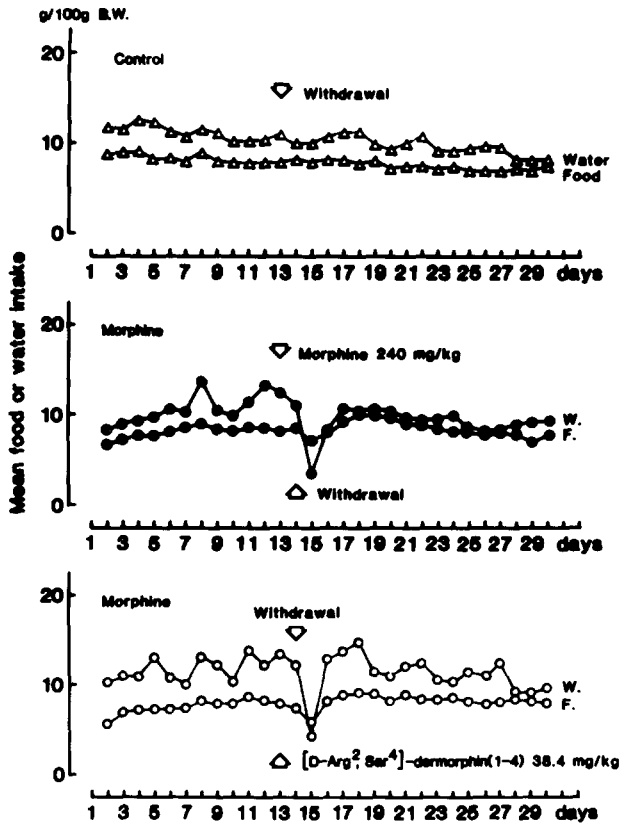

FIG. 5. Changes in the amount of food and water intake after injection of [D-Arg^2, Sar^4]-dermorphin (1–4) in rats chronically administered morphine. See the Method section for injection schedule. The rats were made dependent on morphine by a series of SC injections of morphine administered twice daily. The chronic morphine-treated rats were divided into two groups. One group received [D-Arg^2, Sar^4]-dermorphin, the other morphine on day 13, at the end of treatment. Both groups received no treatment after day 13. Saline-treated animals served as controls. Each point is the mean value obained from 6 rats.

TABLE 1

NALOXONE-PRECIPITATED WITHDRAWAL SEVERITY AFTER CHRONIC TREATMENT OF RATS WITH [D-ARG^2, SAR^4]-DERMORPHIN (1–4) AND MORPHINE

	Number of score		
Signs	Saline	Morphine	[D-Arg^2, Sar^4]-dermorphin (1–4)
Salivation	0	2.6 ± 0.4*	1.7 ± 0.7*
Rhinorrhea	0	1.7 ± 0.7*	0.9 ± 0.6
Lacrimation	0	2.3 ± 0.5*†	0
Urination	0	2.3 ± 0.3*†	0
Diarrhea	0	4.0 ± 0.0*†	0.9 ± 0.4*
Ptosis	0	2.0 ± 0.6*	0.6 ± 0.6
Teeth chatter	0.3 ± 0.3	1.1 ± 0.7†	4.0 ± 0.0*
Swallowing	0	1.4 ± 0.7	0
Tremor	0	3.1 ± 0.4*	2.0 ± 0.4*
Hunchbach posture	0	4.0 ± 0.0*	3.4 ± 0.4*
Piloerection	0	2.0 ± 0.0*	2.0 ± 0.0*
Irritability to handling	0	3.1 ± 0.4*	3.4 ± 0.4*
Reaction to poking	0	3.1 ± 0.4*	1.1 ± 0.6
Jumping	0	0.6 ± 0.6	0
Digging	1.0 ± 0.5	3.7 ± 0.7*	2.6 ± 0.6
Shakes	1.0 ± 0.5	3.1 ± 1.0	4.0 ± 0.7*
Writhing	0	2.9 ± 1.0*	0.9 ± 0.4

The number represents the means ± S.E.M.

The rats were made dependent on [D-Arg^2, Sar^4]-dermorphin (1–4) and morphine by a series of SC injection. The dependent rats were challenged with naloxone (1 mg/kg, IP). See the Method section for injection schedule.

*Significantly different from saline treated rats ($p<0.05$).

†Significantly different from [D-Arg^2, Sar^4]-dermorphin (1–4) treated rats ($p<0.05$).

shakes, digging, tremor, hunchback posture, piloerection, irritability to handling, reaction to poking with a sharp object, salivation, ptosis, and writhing. There were statistically significant differences in 4 withdrawal signs between the two drugs. As shown in Table 1, the naloxone-precipitated withdrawal score of lacrimation, diarrhea and urination of the [D-Arg^2, Sar^4]-dermorphin (1–4)-treated group was significantly lower than that of the morphine-treated group, and the score of teeth chatter was higher in the peptide-treated group.

DISCUSSION

A tetrapeptide dermorphin analog (H-Tyr-D-Arg-Phe-Sar-OH), [D-Arg^2, Sar^4]-dermorphin (1–4) has been recently reported to possess a potent antinociceptive activity following SC administration among a series of D-Arg^2-dermorphin tetrapeptides tested in mice [16]. In the present study, it was confirmed that [D-Arg^2, Sar^4]-dermorphin (1–4) possessed a more potent antinociceptive activity than morphine in a dose-related manner when given SC as measured by the tail-flick test in the rat. [D-Arg^2, Sar^4]-dermorphin (1–4) induced antinociception was readily antagonized by naloxone and duration of its antinociceptive activity was longer than morphine. These results are consistent with our previous paper on mice [16].

In the present study, the characteristics of dependence induced by [D-Arg^2, Sar^4]-dermorphin (1–4) were examined in comparison with those of morphine in the rat. It is well established that repeated administration of morphine results in the development of physical dependence that can be demonstrated following abrupt cessation of morphine [1, 7, 13]. The withdrawal syndrome produced by abrupt cessation of [D-Arg^2, Sar^4]-dermorphine (1–4) includes only a slight diarrhea and body weight loss, though a number of physiological and behavioral signs of spontaneous withdrawal have been reported in chronically morphine-treated rats [1, 7, 13]. The difference in physical dependence between [D-Arg^2, Sar^4]-dermorphin (1–4) and morphine may be partially explained by a slower decrease in peptide blood level because of its longer duration of action. The administration of an opioid antagonist, naloxone to rats given repeated doses of [D-Arg^2, Sar^4]-dermorphin (1–4) evoked a more characteristic withdrawal syndrome than spontaneously occurring

withdrawal. While the profile of withdrawal signs after [D-Arg^2, Sar^4]-dermorphin (1–4) is qualitatively similar to morphine, this tetrapeptide treatment produced a weaker degree of physical dependence as expressed by the cessation or naloxone-induced withdrawal syndrome. Similarly, Broccardo *et al.* [2] found that withdrawal symptoms, precipitated by naloxone treatment were considerably less severe with dermorphin than with morphine when injected into the cerebroventricle. Body weight loss has been proposed as being the most reliable and objective measure of withdrawal in rats [1,7]. In the present experiment, abrupt withdrawal of [D-Arg^2, Sar^4]-dermorphin (1–4) produced a weaker degree of body weight loss than morphine, though the amount of food and water intake was depressed to the same extent. The result in this withdrawal study suggests that the amount of food and water intake should be recorded at the same time with the measurement of body weight. Moreover, the discrepancy is probably due to the result that morphine-treated rats developed severe diarrhea when compared to tetrapeptide-treated rats in the withdrawal test as well as in the naloxone test. This concept is supported by the report [13] that obese mice infused with pancreatic polypeptide developed both diarrhea and weight loss in a dose-dependent fashion. Therefore, the parallel occurrence of diarrhea makes it likely that the weight loss is secondary to the diarrheal illness in morphine-treated rats.

The present results in the naloxone test indicate that tetrapeptide produced physical dependence which was characterized by part of the withdrawal syndrome. However, a naloxone challenge produced diuretic effect in morphine-treated rats in contrast to a lack of diuresis in tetrapeptide-treated rats. The difference of diuretic effect between morphine and tetrapeptide may be explained by vasopressin inhibition, since it has been postulated the antidiuresis induced by acute administration of morphine was mediated through the liberation of vasopressin [4]. In addition, the sign of lacrimation was not observed by precipitation of tetrapeptide withdrawal with naloxone, though it was observed in morphine dependent animals after naloxone treatment. It should be mentioned that lacrimation is a clinically useful indicator of opioid withdrawal in the naloxone test [9].

It seems apparent from the results of naloxone antagonism that [D-Arg^2, Sar^4]-dermorphin (1–4) may act on μ-receptors in the brain. This is supported by our preliminary data that this tetrapeptide inhibited the binding of ^{3}H-naloxone or ^{3}H-dihydromorphine to rat brain membrane (unpublished data). However, it is incomprehensible that a large dose of [D-Arg^2, Sar^4]-dermorphin equal to 64 times the ED_{50} could not substitute for morphine (μ-agonist) in preventing weight loss, regardless of its high affinity for the μ-receptors. Moreover, it should be noted that there were no killed animals when given such a large dose of the peptide. Therefore, there is a possibility that [D-Arg^2, Sar^4]-dermorphin (1–4) may have a cross tolerance with morphine in the lethal experiment. Further studies are needed to clarify the propriety of this consideration.

In conclusion, the present data suggests that morphine and [D-Arg^2, Sar^4]-dermorphin (1–4) differ in their mechanisms of production of physical dependence in the central nervous system. This suggestion is supported by the additional result that [D-Arg^2, Sar^4]-dermorphin (1–4) failed to substitute for morphine in the morphine-dependent rat.

ACKNOWLEDGEMENT

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