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Antinociception and physical dependence produced by [D-Arg^2] dermorphin tetrapeptide analogues and morphine in rats

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1 The antinociceptive effects of [D-Arg^2] dermorphin tetrapeptide analogues, H-Tyr-D-Arg-Phe-Gly-NH_2 and H-Tyr-D-Arg-Phe-β-Ala-OH when administered subcutaneously (s.c.) in rats were measured by the tail-flick test. In addition, the appearance of typical withdrawal signs upon cessation of administration or on subsequent treatment with naloxone were measured after chronic administration of either peptide or morphine.

2 The dose of peptides and of morphine in the physical dependence test was determined from the AD_{50} to inhibit the tail-flick test in rats. Doses from 4 to 64 times the AD_{50} doses were employed in the s.c. administration schedules.

3 The intensity of the antinociception induced by either peptide was greater than that produced by morphine. Moreover, the antinociception induced by the peptides was of much longer duration than that produced by morphine.

4 Abrupt withdrawal after chronic administration of either peptide produced only slight loss of body weight. In contrast, morphine withdrawal produced sharp loss of body weight.

5 Naloxone precipitated withdrawal signs after chronic administration of either peptide were less intense than those after chronic morphine.

6 These results suggest that the antinociception produced by these peptides is more intense and of longer duration than that produced by morphine. It is also interesting to note that the physical dependence produced by these peptides is less marked than that produced by morphine.

Introduction

Dermorphin (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2), is a heptapeptide amide that was isolated (Erspamer & Melchiorri, 1980) and identified (Montecucchi *et al.*, 1981) in the skin of a South American frog, and found to produce potent and long-lasting antinociceptive activity (Broccardo *et al.*, 1981; 1982; Feuerstein & Faden, 1983; Güllner & Kelly, 1983). The antinociceptive effect of dermorphin is known to be more powerful than that of other naturally occurring opioid peptides or of morphine. It has been proposed that the N-terminal tetrapeptide amide (H-Tyr-D-Ala-Phe-Gly-NH_2) is the minimal fragment required for opioid activity, and D-Ala in position 2 in the peptide chain of dermorphin is of crucial importance for opioid activity (Broccardo *et al.*, 1981; De Castiglione *et al.*, 1981; Salvadori *et al.*, 1982b). However, it has been found experimentally that this dermorphin tetrapeptide amide fragment is rather less potent than the parent heptapeptide amide (Broccardo *et al.*, 1981; Erspamer *et al.*, 1981; Salvadori *et al.*, 1982a). The

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antinociceptive effect of dermorphin was completely antagonized by pretreatment with naloxone. In addition, tolerance and physical dependence were consistently less marked with dermorphin than with morphine (Broccardo *et al.*, 1981).

The activity of D-Arg^2 substituted dermorphin tetrapeptide (H-Tyr-D-Arg-Phe-Gly-OH) was more potent than morphine or the dermorphin tetrapeptide (H-Tyr-D-Ala-Phe-Gly-OH) itself (Sasaki *et al.*, 1984; 1985). Kisara *et al.* (1986) studied the activity of [D-Arg^2] dermorphin fragments and showed both that the N-terminal tetrapeptide amide (H-Tyr-D-Arg-Phe-Gly-NH_2) was the most potent fragment and that the N-terminal tripeptide amide (H-Tyr-D-Arg-Phe-NH_2) was the minimal fragment required for opioid activity.

In the present study, we have examined the antinociceptive effects of the [D-Arg^2] dermorphin tetrapeptide analogues, H-Tyr-D-Arg-Phe-Gly-NH_2 and H-Tyr-D-Arg-Phe-β-Ala-OH, administered subcutaneously (s.c.), in the tail-flick test. The appearance of typical withdrawal signs upon cessation of administration or on subsequent treatment with naloxone after chronic administration of either peptide or morphine was also examined.

Method

Male Wistar rats, weighing 160 to 180 g were used in the present study. The animals were housed at 22 ± 2°C with food and water available *ad libitum*. A standard light-dark cycle was maintained with a time regulated light period from 09 h 00 min to 21 h 00 min.

Morphine hydrochloride (Sankyo), naloxone hydrochloride (Endo laboratories) and [D-Arg^2] dermorphin tetrapeptide analogues, H-Tyr-D-Arg-Phe-Gly-NH_2 and H-Tyr-D-Arg-Phe-β-Ala-OH (synthesized by Suzuki *et al.*) were used in these experiments.

The antinociceptive effect was measured by the tail-flick test, adapted from the classical design of D'Amour & Smith (1941). The latency of tail-flick response to heat focused on the tip of the tail was measured with a tail-flick unit (UGO BASIL, model-DS20) as previously described (Kawamura *et al.*, 1983). Tail-flick latencies to thermal stimuli were determined at 15, 30, 45, 60, 90, 120, 180, 240, 300 and 360 min after peptide or morphine administration. Naloxone was administered s.c., 5 min before peptide or morphine administration. A cut-off time of 15 s was imposed on animals failing to remove their tails from light beam to avoid tail tissue damage. The antinociceptive effect for each rat was calculated according to the following equation and represented as % of maximum possible effect (% 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 administration and T_2 was then measured for each animal at various intervals after drug administration. The AD_{50} (antinociceptive dose = 50% MPE) values and their 95% confidence limits were determined by the method of Litchfield & Wilcoxon (1949). These values were calculated from measurements obtained at the time of peak effect after either peptide or morphine administration.

The withdrawal study was modified from the original method (Nakata *et al.*, 1986). Each drug was administered s.c. twice daily (09 h 00 min and 21 h 00 min). Morphine at 6 mg kg^{-1} was administered s.c. twice daily (12 mg kg^{-1} day^{-1}) for the first two days. Subsequently, 12 mg kg^{-1} (24 mg kg^{-1} day^{-1}) on days 3 and 4, 24 mg kg^{-1} (48 mg kg^{-1} day^{-1}) on days 5 and 6, 48 mg kg^{-1} (96 mg kg^{-1} day^{-1}) on days 7 and 8, 96 mg kg^{-1} (192 mg kg^{-1} day^{-1}) on days 9 and 10 were administered. On day 11, the effect of abrupt withdrawal of morphine on body weight and water and food consumption was tested by giving saline. On days 12 and 13, the final dose of morphine at 96 mg kg^{-1} (192 mg kg^{-1} day^{-1}) was re-administered s.c. twice daily. On day 14, abrupt withdrawal was again produced and body weight and water and food consumption were measured following administration of saline. The administration schedule of both peptides was the same as that for morphine; the dose of the peptides increased from 4 to 64 times the AD_{50}. The initial dose of H-Tyr-D-Arg-Phe-Gly-NH_2 at 1.4 mg kg^{-1} was administered s.c. twice daily (2.8 mg kg^{-1} day^{-1}) for the first two days. Subsequently, 2.8 mg kg^{-1} (5.6 mg kg^{-1} day^{-1}) on days 3 and 4, 5.6 mg kg^{-1} (11.2 mg kg^{-1} day^{-1}) on days 5 and 6, 11.2 mg kg^{-1} (22.4 mg kg^{-1} day^{-1}) on days 7 and 8, 22.4 mg kg^{-1} (44.8 mg kg^{-1} day^{-1}) on days 9, 10, 12 and 13 were administered. The initial dose of H-Tyr-D-Arg-Phe-β-Ala-OH at 0.66 mg kg^{-1} was administered s.c. twice daily (1.32 mg kg^{-1} day^{-1}) for the first two days. Subsequently, 1.32 mg kg^{-1} (2.64 mg kg^{-1} day^{-1}) on days 3 and 4, 2.64 mg kg^{-1} (5.28 mg kg^{-1} day^{-1}) on days 5 and 6, 5.28 mg kg^{-1} (10.56 mg kg^{-1} day^{-1}) on days 7 and 8, 10.56 mg kg^{-1} (21.12 mg kg^{-1} day^{-1}) on days 9, 10, 12 and 13 were administered. Control groups were treated with saline.

In the naloxone challenge study, the administration schedule of both peptides and morphine was according to the first 10 days schedule used in the withdrawal study. At 09 h 00 min on day 11, the rats were challenged with naloxone (1 mg kg^{-1}, i.p.). Withdrawal severity was assessed for 15 min following the administration of naloxone. The following

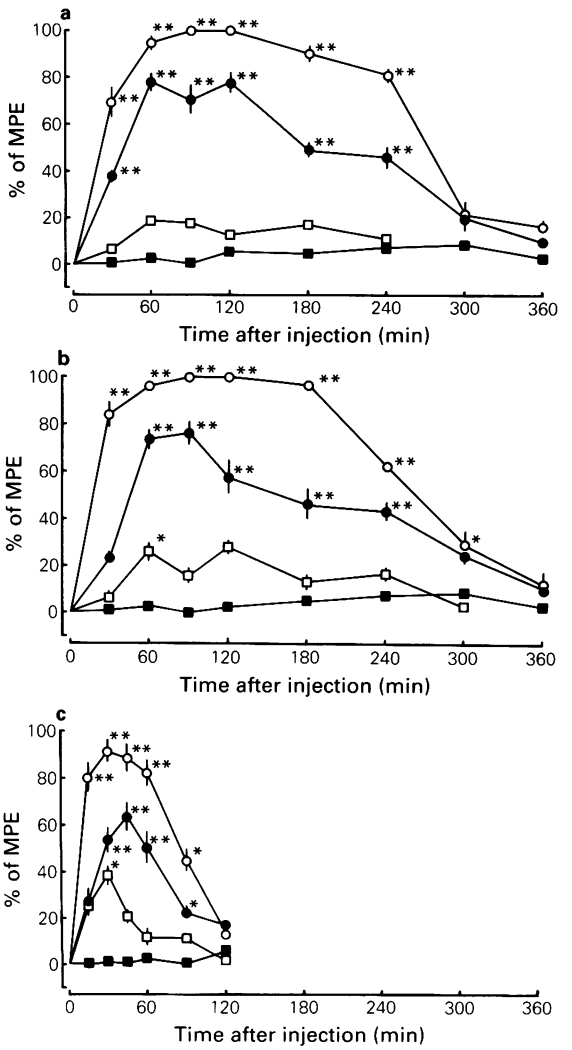

Figure 1 The antinociceptive effects of H-Tyr-D-Arg-Phe-Gly-NH_2 (a), H-Tyr-D-Arg-Phe-β-Ala-OH (b) and morphine (c) when administered subcutaneously as measured by the tail-flick test in rats. (a) H-Tyr-D-Arg-Phe-Gly-NH_2, 2.0 (○), 1.0 (●) and 0.5 (□) mg kg^{-1}. (b) H-Tyr-D-Arg-Phe-β-Ala-OH, 1.0 (○), 0.5 (●) and 0.25 (□) mg kg^{-1}. (c) Morphine, 10 (○), 5 (●) and 2.5 (□) mg kg^{-1}. Control groups (■) were treated with saline. MPE = maximum possible effect (see Methods).) Each point represents the mean, with vertical lines showing s.e.mean, of 6–7 rats in each group. Significant differences from control groups are indicated by $*P < 0.05$ and $**P < 0.01$.

signs were scored by the method of Frederickson & Smits (1973): salivation, rhinorrhoea, lacrimation, urination, diarrhoea, erection, ejaculation, ptosis,

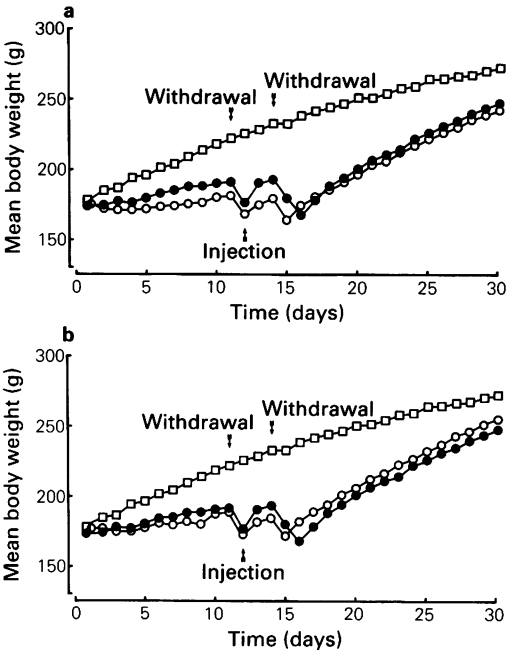

Figure 2 Changes of body weight after abrupt cessation of drug administration in rats chronically treated with H-Tyr-D-Arg-Phe-Gly-NH_2(a) (○), H-Tyr-D-Arg-Phe-β-Ala-OH (b) (○) and morphine (a,b) (●). Control groups (□) were treated with saline. Each point represents the mean, of 6–7 rats in each group.

teeth chatter, swallowing, tremor, hunchback posture, piloerection, irritability to handling, reaction to poking sharp object, escape behaviour (jumping or digging), wet dog shakes, head shakes, foreleg shakes and writhing.

Statistical significance of the data was estimated by analysis of variance (ANOVA) (Haber & Runyon, 1977) with Tukey's test or Dunnett's test.

Results

Antinociceptive effects of test drugs as measured by the tail-flick test

Both peptides and morphine when administered s.c. produced dose-related antinociceptive effects. Maximally effective doses of H-Tyr-D-Arg-Phe-Gly-NH_2 (2.0 mg kg^{-1}) and H-Tyr-D-Arg-Phe-β-Ala-OH (1.0 mg kg^{-1}) produced peak antinociceptive effects 90 to 120 min after administration. The total duration of the effects was 300 to 360 min. A maximally effective dose of morphine (10.0 mg kg^{-1}) produced

its antinociceptive effect at 30 min after administration. The total duration of this effect was 120 min (Figure 1).

The AD_{50} (95% confidence limits) values for H-Tyr-D-Arg-Phe-Gly-NH_2, H-Tyr-D-Arg-Phe-β-Ala-OH and morphine were 0.70 (0.55–0.89), 0.33 (0.25–0.43) and 3.00 (1.38–6.52) mg kg^{-1}, respectively. From these values, the intensity of the effect of H-Tyr-D-Arg-Phe-Gly-NH_2 was 4.3 times, and H-Tyr-D-Arg-Phe-β-Ala-OH was 9.1 times that of morphine. Moreover, the effects of both peptides lasted approximately 3 times longer than those of morphine. The antinociceptive effects of both peptides and morphine were completely antagonized by pretreatment with naloxone (0.5 mg kg^{-1}, s.c.) (data not shown).

Effects of withdrawal on body weight and food and water intake

After chronic administration of either peptide abrupt withdrawal on day 14 produced only a slight loss of body weight (H-Tyr-D-Arg-Phe-Gly-NH_2 produced approximately 7.9% and H-Tyr-D-Arg-Phe-β-Ala-OH approximately 7.1% loss of body weight) at 24 to 48 h post-withdrawal as compared to the weight on the last day of peptide treatment. In contrast, withdrawal of morphine on day 14 produced a sharp loss of body weight (approximately 12.8% at maximum) at 72 h post-withdrawal (Figures 2 and 3).

Water intake was markedly depressed on withdrawal of either peptide or morphine at 24 h. Food intake was also markedly depressed on withdrawal in both peptide-treated groups, but only a slight depression of food intake was seen after withdrawal in the morphine-treated group (Figure 4).

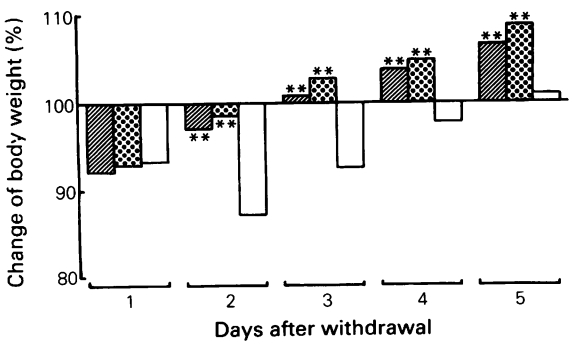

Figure 3 Changes of body weight (%) after withdrawal of either H-Tyr-D-Arg-Phe-Gly-NH_2 (hatched columns), H-Tyr-D-Arg-Phe-β-Ala-OH (stippled columns) or morphine (open columns). Each column represents the mean of 6–7 rats in each group. Significant differences from the morphine treated groups are indicated by $^{**}P < 0.01$.

Naloxone-precipitated withdrawal

Naloxone precipitated withdrawal signs after chronic administration of either peptide were found to be rather less intense than those seen after chronic administration of morphine. Mean withdrawal scores of peptide-treated groups were significantly lower than the mean withdrawal score of the morphine-treated group (Figure 5). Withdrawal in the H-Tyr-D-Arg-Phe-Gly-NH_2-treated group was characterized by irritability to handling and piloerection, while in the H-Tyr-D-Arg-Phe-β-Ala-OH-treated group withdrawal was characterized by ptosis and irritability to handling. In contrast, withdrawal in the group treated with morphine resulted in rhinorrhoea, urination, diarrhoea, ptosis, teeth chatter, swallowing, tremor, hunchback posture, irritability to handling, piloerection and wet dog shakes (Table 1).

Discussion

In a previous study, we examined the structure-activity relationship of [D-Arg^2]-dermorphin tetrapeptide analogues. We showed that the substitution of β-Ala for Gly in the H-Tyr-D-Arg-Phe-Gly-OH peptide chain resulted in greater antinociceptive activity than seen for other [D-Arg^2]-dermorphin tetrapeptide analogues. Peptides were administered intracerebroventricularly (i.c.v.) and antinociceptive effects measured by the tail-pressure test in mice (unpublished data).

In the present study, we have examined the antinociceptive effects of the [D-Arg^2]-dermorphin tetrapeptide analogues, H-Tyr-D-Arg-Phe-Gly-NH_2 and H-Tyr-D-Arg-Phe-β-Ala-OH when administered s.c. and compared the characteristics of dependence induced by these peptides with those of morphine-dependence.

The intensity of the antinociception induced by either peptide was greater than that produced by morphine as measured by the tail-flick test. It is interesting that antinociception in either peptide-treated group was of much longer duration than that in the morphine-treated group. Kisara *et al.* (1986) demonstrated that [D-Arg^2] dermorphin is characterized by a longer duration of action than dermorphin (D-Ala^2 type), which seems to arise from resistance to enzymatic degradation on account of the presence of the D-Arg^2 residue. The same conclusion was drawn from experiments in which the D-Arg^2 substituted dermorphin tetrapeptide (H-Tyr-D-Arg-Phe-Gly-OH) was formed to be more stable than the parent tetrapeptide (H-Tyr-D-Ala-Phe-Gly-OH) to the cleavage both by aminopeptidase M and carboxypeptidase Y (Sasaki *et al.*, 1985). Thus, D-Arg

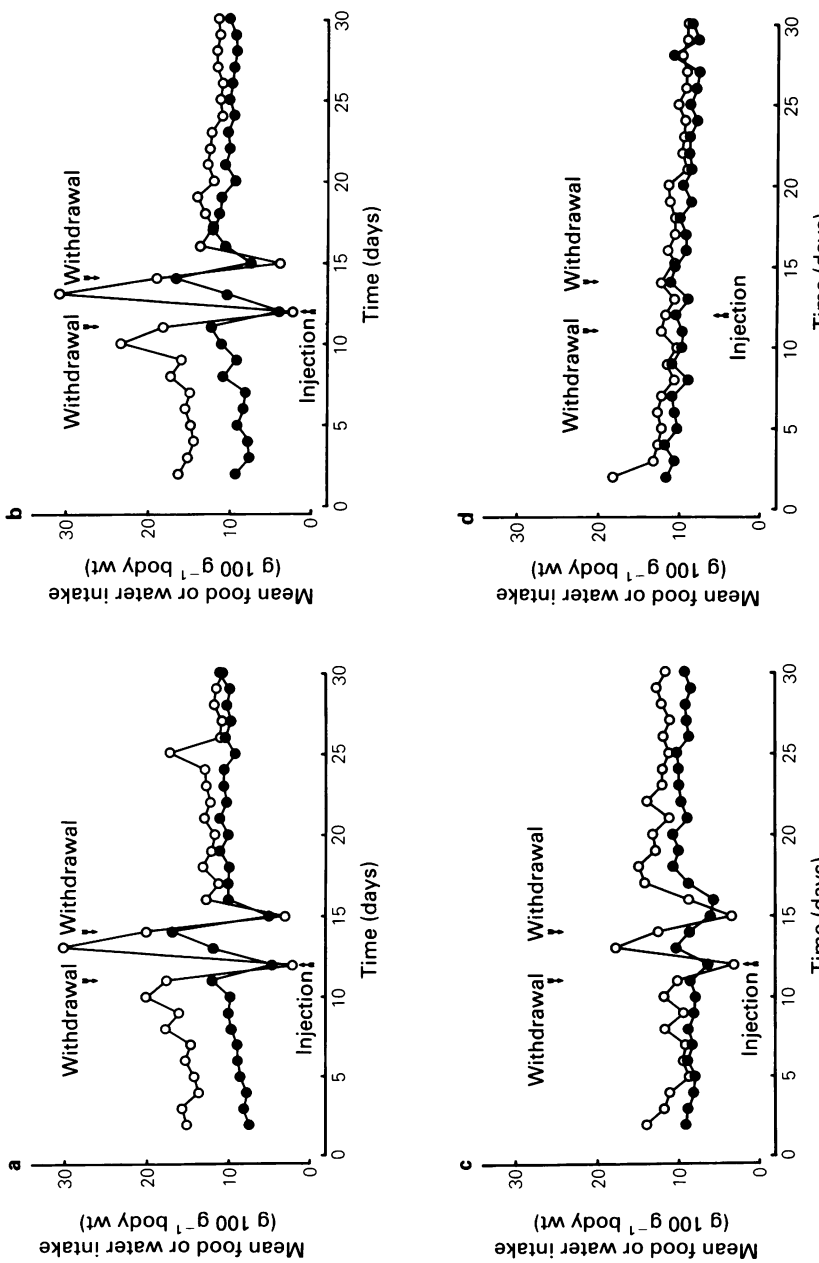

Figure 4 Changes in food (●) and water (○) intake after abrupt cessation of drug administration in rats chronically treated with either H-Tyr-D-Arg-Phe-Gly-NH_2 (a), H-Tyr-D-Arg-Phe-β-Ala-OH (b) or morphine (c). Control groups were treated with saline (d). Each point represents the mean of 6–7 rats in each group.

in position 2 of [D-Arg^2] dermorphin fragments is of crucial importance for opioid activity.

Sato *et al.* (1987) have recently shown that other [D-Arg^2] dermorphin tetrapeptide analogues, H-Tyr -D-Arg-Phe-Gly-OH and H-Tyr-D-Arg-Phe-Sar-OH, have high affinity for μ-type opioid receptors in the radioreceptor assay utilizing [^{3}H]-naloxone as the opioid receptor ligand. Therefore, antinociceptive activity of these analogues has been considered to be mainly due to the specific interaction with opioid receptors in the central nervous system. From the present results, antinociception induced by both peptides was completely antagonized by pretreatment with naloxone (0.5 mg kg^{-1}, s.c.), suggesting that they may have high affinity for opioid receptors in the central nervous system.

In the present withdrawal test, characteristics of dependence induced by both peptides were compared with the characteristics of morphine-induced dependence. 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 (Akera & Brody, 1968; Goode, 1971; Mordes *et al.*, 1982). The withdrawal syndrome produced by

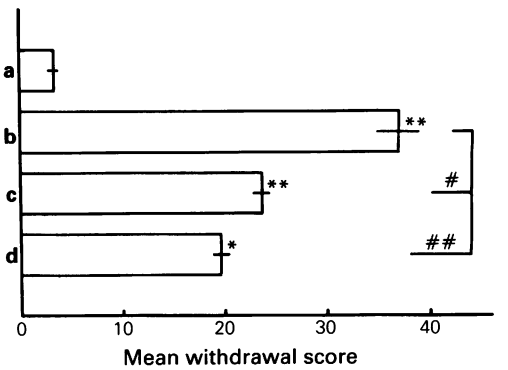

Figure 5 Naloxone-precipitated withdrawal score after chronic administration of (b) morphine, (c) H-Tyr-D-Arg-Phe-Gly-NH_2 and (d) H-Tyr-D-Arg-Phe-β-Ala-OH. (a) Control groups were treated with saline. Each column represents the total mean of each withdrawal sign, with vertical lines showing s.e.mean, of 6–7 rats in each group. Significant differences from control groups are indicated by $^*P < 0.05$, $^{**}P < 0.01$. Significant differences from morphine-treated groups are indicated by $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

Table 1 Naloxone-precipitated withdrawal severity after chronic administration of test drugs

		Number of score		
Signs	*Saline*	*H-Tyr-D-Arg-Phe-Gly-NH_2*	*H-Tyr-D-Arg-Phe-β-Ala-OH*	*Morphine*
Salivation	0	0	0	0
Rhinorrhoea	0	0.9 ± 0.4	1.1 ± 0.4	2.0 ± 0.5*
Lacrimation	0	0.6 ± 0.4	0	0.8 ± 0.4
Urination	0.9 ± 0.4	2.3 ± 0.3	1.7 ± 0.3	2.8 ± 0.4*
Diarrhoea	0	1.7 ± 0.5	1.7 ± 0.3	2.5 ± 0.5*
Ptosis	0.3 ± 0.3	2.3 ± 0.7	2.9 ± 0.4*	2.8 ± 0.4*
Teeth chatter	0	0.9 ± 0.4†	0.9 ± 0.4†	3.8 ± 0.2*
Swallowing	1.1 ± 0.4	2.0 ± 0.6	1.7 ± 0.3	3.3 ± 0.4*
Tremor	0	1.1 ± 0.4	0.6 ± 0.4†	2.8 ± 2.4*
Hunchback posture	0	1.4 ± 0.4	1.4 ± 0.4	2.3 ± 0.3*
Irritability to handling	0	3.8 ± 0.3*	3.4 ± 0.4*	2.8 ± 0.4*
Reaction to poking	0	0	0	0.8 ± 0.4
Piloerection	0	2.0 ± 0.0*	0.6 ± 0.4†	2.5 ± 0.3*
Ejaculation	0	0.3 ± 0.3	0	1.0 ± 0.5
Erection	0	0	0.3 ± 0.3	0.3 ± 0.3
Jumping	0	0.6 ± 0.4	0.3 ± 0.3	0.3 ± 0.3
Digging	0	0	0	0.3 ± 0.3
Wet dog shakes	0.3 ± 0.3	0.9 ± 0.4	0.6 ± 0.4	2.3 ± 0.5*
Head shakes	0.6 ± 0.6	0.3 ± 0.3	0.6 ± 0.4	0
Foreleg shakes	0.3 ± 0.3	0.3 ± 0.3	0.6 ± 0.4	1.3 ± 0.5
Yawning	0	0	0	0
Writhing	0	0.6 ± 0.4	0.6 ± 0.4	1.5 ± 0.6

The data represent means ± s.e.mean.
* Significantly different from saline-treated rats ($P < 0.05$).
† Significantly different from morphine-treated rats ($P < 0.05$).

abrupt cessation of either peptide included only a slight diarrhoea and loss of body weight, in contrast to the considerable number of physiological and behavioural signs of spontaneous withdrawal that have been demonstrated in rats chronically treated with morphine (Akera & Brody, 1968; Goode, 1971; Mordes *et al.*, 1982). This difference between the peptides and morphine may be partially explained by a slower decline in blood level of peptide resulting from their longer duration of action. Body weight loss has been proposed as being the most reliable and objective measure of withdrawal in rats (Akera & Brody, 1968; Goode, 1971). In the present experiments, abrupt withdrawal in groups treated with either peptide produced a smaller body weight loss than withdrawal in the morphine-treated group. However, food and water intake of peptide-treated groups was more markedly depressed than in the morphine-treated group. This discrepancy is probably due to the fact that the morphine-treated group developed more severe diarrhoea than the peptide-treated groups both after abrupt drug withdrawal and during naloxone-precipitated withdrawal. This concept is supported by the finding that obese mice infused with pancreatic polypeptide developed both diarrhoea and weight loss in a dose-dependent fashion (Mordes *et al.*, 1982). Therefore, the parallel occurrence of diarrhoea and weight loss makes it likely that weight loss is secondary to diarrhoea in the morphine-treated group.

Results of the naloxone challenge test indicated that both peptides produced physical dependence which was characterized by part of the withdrawal syndrome. However, naloxone challenge produced a diuretic effect in the morphine-treated group which was not seen in the groups treated with the peptides. This difference may be explained by vasopressin inhibition, since it has been postulated that the antidiuresis induced by acute administration of morphine is mediated through the liberation of vasopressin (De Bode, 1944). In addition, lacrimation was not observed when withdrawal from either peptide or morphine was precipitated with naloxone. It should be mentioned that lacrimation is a clinically useful indicator of opioid withdrawal in the naloxone test (Judson *et al.*, 1980).

In conclusion, these results suggest that the opioid receptor-mediated antinociceptive effects of [D-Arg^2] dermorphin tetrapeptide analogues are more potent and of longer duration than the antinociceptive effect of morphine. Furthermore, it is interesting to note that the physical dependence produced by these analogues is less intense than that produced by morphine.

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