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Dermorphin analogues containing D-kyotorphin: structure-antinociceptive relationships in mice

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1 The antinociceptive effects of synthetic dermorphin and its analogues containing D-Arg in position 2 injected into the lateral cerebroventricle were examined in conscious mice.

2 Intracerebroventricular (i.c.v.) administration of dermorphin and [D-Arg^2] dermorphin produced potent and long-lasting antinociceptive activity as assayed by the tail-pressure test.

3 Dermorphin and [D-Arg^2] dermorphin were 210 and 52 times more potent than morphine, respectively.

4 The antinociceptive effects produced by these heptapeptides were antagonized by a low dose (0.5 mg kg^{-1}, i.p.) of the opioid antagonist naloxone.

5 The ED_{50} values for [D-Arg^2] dermorphin (1–6), (1–5) and (1–4) were not significantly different from that for [D-Arg^2] dermorphin. The potency of the shortest fragment, [D-Arg^2] dermorphin (1–2) was found to possess a severely reduced activity, whilst [D-Arg^2] dermorphin (1–3) maintained activity and was 10 times more potent than morphine.

6 [D-Arg^2] dermorphin analogues showed almost identical effects when tested on the electrically-induced contractions of the guinea-pig isolated ileum.

7 These results led us to conclude that the presence of the N-terminal tripeptide in the structure of [D-Arg^2] dermorphin is of crucial importance for the manifestation of the full intrinsic opioid-like antinociceptive activity of [D-Arg^2] dermorphin, which is presumably mediated through opioid receptors in the brain.

Introduction

Since the isolation and identification of dermorphin in skin extracts of the Brazilian frog (Montecucchi *et al.*, 1981a,b), evidence has accumulated that several other naturally occurring and synthetic peptides have narcotic-like pharmacological activity (De Castiglione *et al.*, 1981; Broccardo *et al.*, 1981; Sato *et al.*, 1984a; 1985). The sequence of dermorphin was determined to be Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2 (Montecucchi *et al.*, 1981a) and it was found to have pronounced opioid antinociceptive activity (Broccardo *et al.*, 1981; Sasaki *et al.*, 1984; Sato *et al.*, 1984b). Opioid binding studies as well as investigations on the electrically stimulated longitudinal muscle of the guinea-pig ileum and the mouse vas deferens preparation confirmed the opioid-like activity (Broccardo *et al.*, 1981; Glaser *et al.*, 1981; Melchiorri *et al.*, 1982). Another type of brain opioid peptide, kyotorphin, that essentially does not bind to opioid receptors, was introduced by Takagi *et al.* (1979a). The antinociceptive action produced by intracisternal or intracerebroventricular (i.c.v.) administration of kyotorphin (L-Tyr-L-Arg) can be prolonged by the use of an analogue (L-Tyr-D-Arg) (Takagi *et al.*, 1979a; Sakurada *et al.*, 1982). The present study was undertaken in order to investigate the effect of substituting D-Arg for D-Ala in position 2 in the peptide chain of dermorphin, and to determine the smallest sequential analogue of [D-Arg^2] dermorphin able to exhibit full activity.

Methods

Male mice (Std-ddy) weighing 22 to 25 g and Hartley guinea-pigs weighing 250 to 300 g were housed in group cages in a temperature- and light-controlled room (22 ± 2°C, 12 h light starting at 09 h 00 min). All mice and guinea-pigs had free access to laboratory chow and tap water, except during experiments which were performed between 10 h 00 min and 18 h 00 min.

Antinociceptive activity was measured by the

tail-pressure method as previously described (Sakurada *et al.*, 1982). Briefly, mechanical pressure was applied to the base of the tail at a rate of 20 mmHg s^{-1} and biting or struggling behaviour in mice to which pressure was applied mechanically was an indication of response threshold. The trials were terminated at the level of 200 mmHg to prevent tail tissue damage. For the tail pressure assay, the mean ± s.e. of the pressure level was plotted. To obtain the dose-response curve, the dose was plotted against % maximum possible effect (% MPE) calculated using the following equation % MPE = (P2 − P1/200 − P1) × 100 where P1 is the response pressure before peptide injection (mmHg) and P2 is the response pressure (mmHg) after peptide injection (mmHg). The peptides were dissolved in Ringer solution and administered with a Hamilton syringe into the lateral ventricle of unanaesthetized mice in a volume of 10 µl. To evaluate opioid specificity of antinociception induced by the peptides, naloxone (0.5 mg kg^{-1}) was given intraperitoneally (i.p.) 5 min before i.c.v. administration of the peptides. Naloxone was prepared for i.p. injection such that the amount given was in a volume of 0.1 ml of 0.9% w/v NaCl solution (saline) per 10 g of body weight. The ED_{50} values, their 95% confidence limits, and the significance of the potency ratio between ED_{50} values were determined by the method of Litchfield & Wilcoxon (1949).

Guinea-pigs were killed by a blow to the head and the ileum was immediately taken from the terminal portion after a 10 cm length nearest the ileocaecal junction had been discarded. The segments (2–3 cm long) of the ileum were mounted longitudinally in a 10 ml isolated tissue bath containing Krebs-Henseleit solution of the following composition (mM): NaCl 117.56, KCl 5.36, $MgSO_4$ 0.57, $CaCl_2$ 1.90, $NaHSO_4$ 0.90, $NaHCO_3$ 23.81 and glucose 11.10. The tissues were maintained at 37°C, aerated with 95% O_2–5% CO_2 and stimulated at a frequency of 0.1 Hz, duration 0.5 ms and potential 10 V, provided by an electronic stimulator (Nihon Kohden, Japan). The peptides were added to the bath in a non-cumulative fashion to evaluate inhibition of the field-stimulated twitch response. The height of the guinea-pig ileum twitch response was measured before and after peptide challenge. The % inhibition of twitch height was determined. A minimum of 3 doses was used for each tissue preparation.

Compounds used were: dermorphin, [D-Arg^2] dermorphin, [D-Arg^2] dermorphin (1–6), [D-Arg^2] dermorphin (1–5), [D-Arg^2] dermorphin (1–4), [D-Arg^2] dermorphin (1–3), [D-Arg^2] dermorphin (1–2), morphine hydrochloride (Takeda Chemical Industries) and naloxone hydrochloride (Endo Laboratories). Dermorphin and its analogues were synthesized by the conventional liquid phase methods in our laboratory.

Duncan's procedure for multiple comparisons was used to analyse the overall patterns of results for antinociceptive test. Individual *t* tests or analyses of variance were carried out as necessary. A *P* value of less than 0.05 was considered significant.

Results

Antinociceptive effects of dermorphin and its analogue, [D-Arg^2] dermorphin

Figure 1 shows the time course of the effects of dermorphin and [D-Arg^2] dermorphin. Dermorphin, given to mice at doses of 8.4, 6.5 and 4.8 pmol (i.c.v.), produced a dose-related antinociceptive effect. Maximally effective doses of dermorphin gave peak antinociceptive activity 10 min post-injection with diminishing effectiveness over about 90 min. The effective antinociceptive dose, ED_{50} (95% confidence limits) was 5.7 (4.8–6.8) pmol per mouse (Table 3). I.c.v. administration of Ringer solution was without effect. The introduction of D-Arg into position 2 of the dermorphin structure resulted in a decrease of antinociceptive activity. The ED_{50} value for [D-Arg^2] dermorphin was 23.0 (16.4–32.3) pmol per mouse (Table 3). Hence, the potency of dermorphin was significantly higher than that of [D-Arg^2] dermorphin with respect to their ED_{50} values. [D-Arg^2] dermorphin exhibited the same time of peak effect as dermorphin, whereas the duration of the antinociceptive effect with the maximum dose (68.4 nmol) of dermorphin was longer than that with the maximum dose (8.4 nmol) of [D-Arg^2] dermorphin.

When a low dose (0.5 mg kg^{-1}) of naloxone, an opioid antagonist, was injected intraperitoneally 5 min before the administration of both heptapeptides, the antinociceptive effects were antagonized completely (Table 1).

Antinociceptive effect of the N-terminal [D-Arg^2] dermorphin fragments

The N-terminal fragment, [D-Arg^2] dermorphin (1–6), produced by removing a single amino acid residue within the [D-Arg^2] dermorphin molecule, exhibited a higher antinociceptive activity than [D-Arg^2] dermorphin (Figure 2), although there was no statistically significant difference between their ED_{50} values (Table 3). The N-terminal pentapeptide, [D-Arg^2] dermorphin (1–5) showed approximately the same antinociceptive potency as [D-Arg^2] dermorphin. The relative potency of [D-Arg^2] dermorphin (1–4)-tetrapeptide was approximately 2 times more than that of [D-Arg^2] dermorphin, but there was no significant difference between their ED_{50} values.

The antinociceptive activity of the tripeptide was significantly less than of [D-Arg^2] dermorphin and its

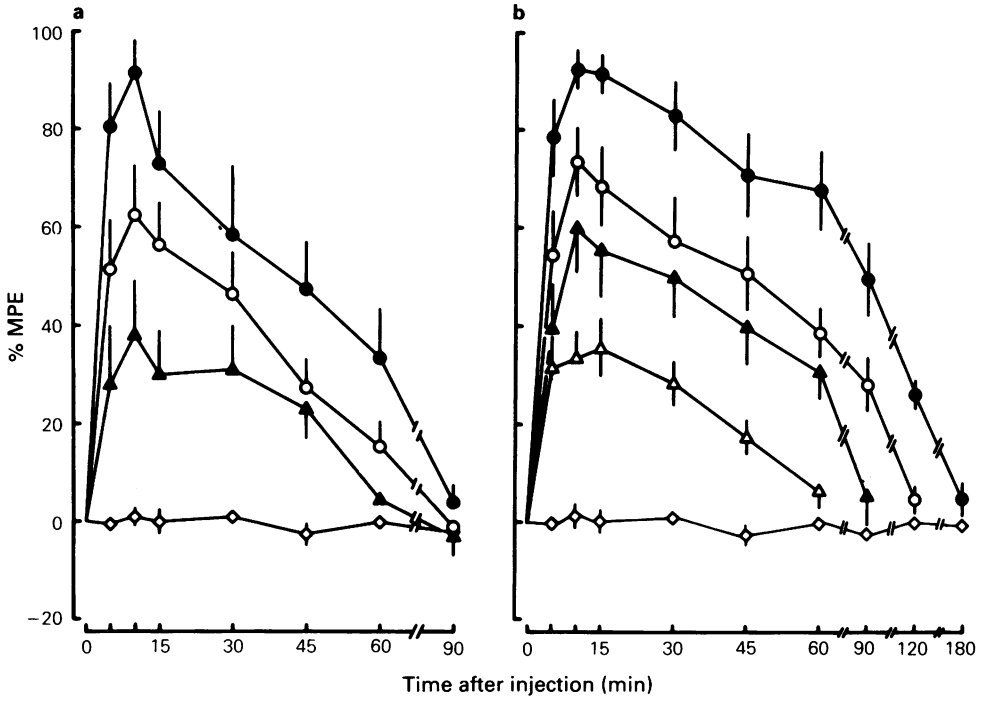

Figure 1 The time courses of the antinociceptive effects of different concentrations of (a) dermorphin and (b) [D-Arg^2] dermorphin administered intracerebroventricularly in mice. Dermorphin, 4.8 (▲), 6.5 (○) and 8.4 (●) pmol. [D-Arg^2] dermorphin, 14.2 (△), 23.9 (▲), 40.5 (○) and 68.4 (●) pmol. Each point represents the mean, with vertical lines showing s.e.mean, of ten mice in each group. Control groups (◇) were treated with Ringer solution. MPE = maximum possible effect (see Methods).

Table 1 Effect of naloxone on the antinociceptive activities of [D-Arg^2] dermorphin and its fragments in mice

Treatments (dose:pmol per animal)	*Changes in threshold (%)* *10 min*	*15 min*
Saline/Ringer	2.0 ± 1.0	11.5 ± 0.8
Saline/[D-Arg^2] dermorphin (40.5)	73.7 ± 6.9	68.3 ± 8.0
Naloxone/[D-Arg^2] dermorphin (40.5)	21.7 ± 5.6*	6.9 ± 5.1*
Saline/[D-Arg^2] dermorphin (1–6) (52.6)	91.3 ± 7.0	85.0 ± 9.1
Naloxone/[D-Arg^2] dermorphin (1–6) (52.6)	32.8 ± 10.4*	40.9 ± 12.0*
Saline/[D-Arg^2] dermorphin (1–5) (52.6)	73.7 ± 8.9	62.5 ± 7.9
Naloxone/[D-Arg^2] dermorphin (1–5) (52.6)	10.3 ± 4.3*	17.8 ± 8.4*
Saline/[D-Arg^2] dermorphin (1–4) (31.1)	83.7 ± 7.8	92.5 ± 5.7
Naloxone/[D-Arg^2] dermorphin (1–4) (31.1)	17.0 ± 4.7*	19.8 ± 4.6*
Saline/[D-Arg^2] dermorphin (1–3) (725.3)	92.1 ± 5.2	81.6 ± 9.4
Naloxone/[D-Arg^2] dermorphin (1–3) (725.3)	25.1 ± 5.2*	18.9 ± 5.6*
Saline/[D-Arg^2] dermorphin (1–2) (81600.0)	76.8 ± 9.9	59.0 ± 8.6
Naloxone/[D-Arg^2] dermorphin (1–2) (81600.0)	44.7 ± 6.6*	35.2 ± 6.0*
Naloxone 1 mg kg^{-1}/[D-Arg^2] dermorphin (1–2) (81600.0)	43.3 ± 7.3*	30.0 ± 5.5*

Naloxone (0.5 mg kg^{-1} i.p.) or saline was given 5 min before the i.c.v. administration of [D-Arg^2] dermorphin or its analogues. Nociceptive responses were determined 30 min before, and 10 and 15 min after the injection of each peptide. **$P < 0.01$, compared to each control group (saline plus peptide).

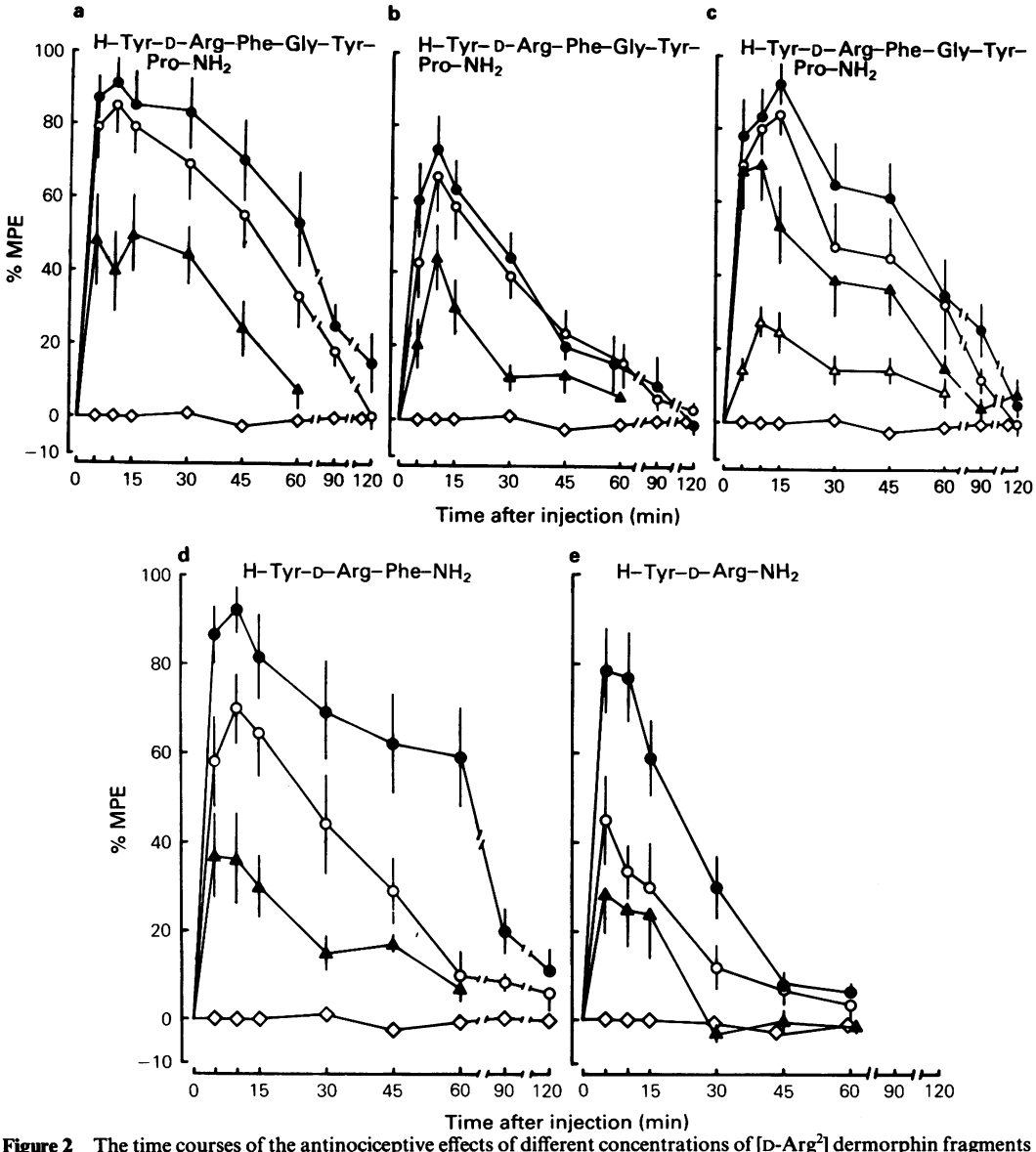

Figure 2 The time courses of the antinociceptive effects of different concentrations of [D-Arg^2] dermorphin fragments administered intracerebroventriculatly in mice. (a) [D-Arg^2] dermorphin (1–6), 10.6 (▲), 31.1 (○) and 52.6 (●) pmol. (b) [D-Arg^2] dermorphin (1–5), 18.4 (△), 31.1 (○) and 52.6 (●) pmol. (c) [D-Arg^2] dermorphin (1–4), 6.5 (△), 10.6 (△), 18.4 (○) and 31.1 (●) pmol. (d) [D-Arg^2] dermorphin (1–3), 151.3 (▲), 429.2 (○) and 725.3 (● pmol. (e) [D-Arg^2] dermorphin (1–2), 28.6 (▲), 48.3 (○) and 81.6 (●) nmol. Each point represents the mean, with vertical lines showing s.e.mean, of ten mice in each group. Control groups (◇) were treated with Ringer solution.

N-terminal fragments, hepta-, penta- and tetrapeptides, although the tripeptide still maintained a higher activity than morphine. The dipeptide, Tyr-D-Arg amide, in which phenylalanine in position 3 of the N-terminal tripeptide was removed, showed a marked decrease in antinociceptive activity.

Pretreatment with naloxone (0.5 mg kg^{-1}, i.p.) resulted in nearly complete antagonism of the antinociceptive effects produced by all of the fragments of [D-Arg^2] dermorphin except those of the dipeptide. The dipeptide-induced antinociception was significantly reversed by low doses (0.5 and 1.0 mg kg^{-1}, i.p.) of

Table 2 Antinociceptive activities produced by intracerebroventricular administration of [D-Arg^2] dermorphin and its fragments, as measured by the tail pressure test in mice

Compound	*ED_{50}* (pmol per mouse)[a]	*Relative potency*[b]
[D-Arg^2] dermorphin	23.0 (16.4–32.3)	100.0
[D-Arg^2] dermorphin (1–6)	12.5 (7.4–21.3)	184.0
[D-Arg^2] dermorphin (1–5)	20.2 (12.7–31.6)	113.9
[D-Arg^2] dermorphin (1–4)	10.3 (7.3–14.6)	223.3
[D-Arg^2] dermorphin (1–3)	210.0 (148.8–304.5)*	11.0
[D-Arg^2] dermorphin (1–2)	47000.0 (3032.0–7285.0)*	0.5
Dermorphin	5.7 (4.8– 6.8)*	403.5
Morphine	1200.0 (700.0–2200.0)*	1.9

[a]ED_{50} values were calculated from the values obtained at the time of peak effect, 95% confidence limits are given in parentheses.
[b]Potencies are relative to [D-Arg^2] dermorphin (= 100) on a molar basis.
*The ED_{50} value was significantly different from that obtained with [D-Arg^2] dermorphin ($P < 0.05$).

naloxone, although the degree of antagonism was less than 50% even with a dose of 1.0 mg kg^{-1} naloxone (Table 1).

Effect of [D-Arg^2] dermorphin and its N-terminal fragments on contractions of the guinea-pig ileum produced by field stimulation

[D-Arg^2] dermorphin inhibited the field-stimulated response of the guinea-pig ileum (Table 2). The relative potency of [D-Arg^2] dermorphin hexa- and pentapeptides was lower than that of [D-Arg^2] dermorphin, but their IC_{50} values were not significantly different from that for [D-Arg^2] dermorphin. However, the N-terminal tetrapeptide was found to be the most potent of the [D-Arg^2] dermorphin fragments; the tetrapeptide was approximately 9 times more active than [D-Arg^2] dermorphin, hexapeptide. A tripeptide fragment had the same activity as morphine, approximately 40% of the potency of [D-Arg^2] dermorphin.

Discussion

Dermorphin, a heptapeptide with exceedingly potent and long-lasting opioid activity is the first example of a new class of opioid peptides occurring in amphibian skin. With regard to its antinociceptive activity, dermorphin is more potent than any other naturally occurring opioid peptide and most of the synthetic opioid peptides. In this paper, we describe an attempt to obtain a dermorphin analogue with the minimal structure sufficient for dermorphin-like opioid activity in order to determine the active core in the structure of a dermorphin analogue.

Earlier studies showed that the substitution of the second position of enkephalins by a D-Arg residue leads to a marked enhancement of antinociceptive activity as compared with enkephalins with a L-amino acid residue in position 2 (Morley, 1980; Amano *et al.*, 1984). However, such pronounced enhancement was not universally observed following the substitution of enkephalins by D-Ala as assayed in the tail-pinch test (Amano *et al.*, 1984). A similar phenomenon has been shown in the case of the dipeptide kyotorphin (Takagi *et al.*, 1979a).

Of special interest is the present finding that [D-Arg^2] dermorphin (1–4), a N-terminal tetrapeptide was the most potent peptide among the analogues of [D-Arg^2] dermorphin and the potency of the tetrapeptide amide was twice that of [D-Arg^2] dermorphin, as measured by the *in vivo* tail-pressure test. The potency increase on the *in vitro* stimulated guinea-pig ileum assay was much more apparent than in the *in vivo* assay. It was surprising that the removal of three C-terminal amino acids from the dermorphin molecule enhanced activity relative to [D-Arg^2] dermorphin. This favourable phenomenon was not seen in structure-activity studies with dermorphin containing the D-amino acid residue [D-Ala^2], a feature which is unique among naturally occurring peptides of non-bacterial origin (Broccardo *et al.*, 1981). It was concluded that the minimum length of the dermorphin molecule required for full opioid activity was the N-terminal tetrapeptide (Broccardo *et al.*, 1981). In contrast to the results of this structure activity-study with dermorphin, the present data obtained in both *in vivo* and *in vitro* assays indicate that the [D-Arg^2] dermorphin tripeptide is the minimum sequence necessary at the N-terminal in order to produce full dermorphin activity. It was also found that the antinociceptive effect, following administration by the i.c.v. route, was decreased by substituting D-Arg for D-Ala in position 2 of dermorphin. This result may support the hypothesis that the D-Ala^2 residue is of crucial importance for the opioid activity of dermor-

Table 3 IC_{50} values of [D-Arg^2] dermorphin and its fragments on the electrically evoked contractions of the guinea-pig ileum

Compound	*IC_{50}* (nmol)[a]	*Relative potency*[b]
[D-Arg^2] dermorphin	92.0 (51.1–165.6)	100.0
[D-Arg^2] dermorphin (1–6)	190.0 (101.1–357.2)	48.4
[D-Arg^2] dermorphin (1–5)	160.0 (83.3–307.2)	57.5
[D-Arg^2] dermorphin (1–4)	10.8 (5.1–22.7)*	851.9
[D-Arg^2] dermorphin (1–3)	240.0 (121.8–472.8)	38.3
[D-Arg^2] dermorphin (1–2)	44000.0 (24175.8–80080.0)*	0.2
Dermorphin	3.5 (2.0–6.1)*	2628.6
Morphine	270.0 (160.7–453.6)	34.1

[a]IC_{50} values were calculated from a log-probit plot of the inhibitory percentage, 95% confidence limits are given in parentheses.
[b]Potencies are relative to [D-Arg^2] dermorphin (= 100) on a molar basis.
*The IC_{50} values are significantly different from that obtained with [D-Arg^2] dermorphin ($P < 0.05$).

phin (Broccardo *et al.*, 1981). However, [D-Arg^2] dermorphin is characterized by a longer duration of action than dermorphin, which seems to arise from resistance to enzymatic breakdown on account of the presence of the D-Arg^2 residue. The same conclusion is drawn from the experimental result that the D-Arg^2-substituted dermorphin tetrapeptide (Tyr-D-Arg-Phe-Gly-OH) is more stable than the parent tetrapeptide (Tyr-D-Ala-Phe-Gly-OH) to cleavage both by aminopeptidase M and carboxypeptidase Y (Sasaki *et al.*, 1985). Herewith, it should be mentioned that recent results obtained in our laboratory revealed a different degree of potency between dermorphin and [D-Arg^2] dermorphin when administered i.c.v. rather than s.c. The antinociceptive potency of [D-Arg^2] dermorphin was found to be higher, compared to that of dermorphin, when administered s.c. (Sasaki *et al.*, 1985; Suzuki *et al.*, 1985). This suggests that the central action of these substances after systemic administration would be determined not only by their susceptibility to enzymatic attack, but also by their distribution and ability to enter or leave the central nervous tissue. The antinociceptive effects of dermorphin and [D-Arg^2] dermorphin (including its fragments, with the exception of Tyr-D-Arg amide) were antagonized completely by a low dose of the opioid antagonist naloxone. Moreover, like other opioid peptide analgesics, the potent inhibitory activity of these peptides was demonstrated using the guinea-pig ileum preparation. These results suggest that [D-Arg^2] dermorphin and its analogues induce antinociception mainly via an interaction with an opioid binding site. The finding by Glaser *et al.* (1981) that dermorphin is less potent than the enkephalins in inhibiting the specific binding of [^{3}H]-Leu enkephalin to hybrid cell membranes, which appear to possess δ-type (Chang & Cuatrecasas, 1979) or enkephalin receptors (Wahlström *et al.*, 1977), argues against an action at this type of site. Additionally, our preliminary results indicate that dermorphin, dermorphin (1–4), [D-Arg^2] dermorphin and its N-terminal tetrapeptide inhibit the binding of [^{3}H]-naloxone to crude rat brain membranes (unpublished data).

A dipeptide is the smallest peptide to exhibit opioid-like antinociceptive activity (Takagi *et al.*, 1979a; Vavrek *et al.*, 1981; Sakurada *et al.*, 1982). Much higher doses of Tyr-D-Arg amide (D-kyotorphin amide) were required, however, to obtain the ED_{50} and IC_{50} values in the presently described assay system. Moreover, this dipeptide action was not reversed completely by a low dose of naloxone unlike that of the other fragments of [D-Arg^2] dermorphin. These results suggest that the mechanism of action of D-kyotorphin amide (L-Tyr-L-Arg-NH_2) may be similar to that of kyotorphin (L-Tyr-L-Arg), though D-kyotorphin amide was much more potent than kyotorphin, equieffective to D-kyotorphin, and less potent than cyclo (N-methyl-Tyr-Arg), Tyr-D-Arg-OMe and Tyr (Et)-D-Arg-OMe (Sakurada *et al.*, 1982; 1984; Kawamura *et al.*, 1983; Sato *et al.*, 1985). It is apparent from the present and previous results that D-kyotorphin amide-induced antinociception may in part involve the endogenous opioid system. The antinociceptive action of D-kyotorphin amide may possibly be explained on the basis of [Met] enkephalin release, since kyotorphin and D-kyotorphin have been shown to induce [Met] enkephalin release (Takagi *et al.*, 1979b; 1982).

In summary, the present data, using the fragments of [D-Arg^2] dermorphin truncated at the C-terminal moiety, emphasize the important contribution of the N-terminal tripeptide (Tyr-D-Arg-Phe-NH_2) in the chemical structure of [D-Arg^2] dermorphin, to the manifestation of its full antinociceptive activity.

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