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PHARMACOLOGICAL DATA ON DERMORPHINS, A NEW CLASS OF POTENT OPIOID PEPTIDES FROM AMPHIBIAN SKIN

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1 Dermorphin and Hyp^6-dermorphin are the first representatives of a new class of potent opioid peptides occurring in amphibian skin. They present the unique feature of having a D-Ala residue incorporated in the peptide molecule.

2 Dermorphin displayed a potent depressive action on electrically stimulated contractions of the guinea-pig ileum and mouse vas deferens preparations. Dermorphin was respectively 57,294, 18 and 39 times more potent than Met-enkephalin, Leu-enkephalin, β-endorphin, and morphine on the guinea-pig ileum opiate receptors. On the vas deferens receptors, dermorphin was about as potent as the enkephalins and 40 times more potent than morphine. Naloxone was a powerful antagonist to dermorphin in both preparations.

3 Dermorphin produced potent and long-lasting analgesia in mice by intravenous injection, and in rats by intracerebroventricular injection, the ED_{50} being here of the order of 13–23 pmol/rat. Morphine was 752 and 2170 times less potent, depending on the analgesia test used. At high intracerebroventricular doses analgesia was accompanied by catalepsy.

4 Intracerebroventricular infusion of dermorphin induced development of tolerance and precipitation of withdrawal symptoms upon administration of naloxone. Both tolerance and physical dependence were consistently less marked with dermorphin than with morphine.

5 The minimum sequence requirement for full dermorphin activity was represented by the N-terminal tetrapeptide. The presence of the D-Ala^2 residue was of crucial importance.

Introduction

Methanol extracts of the skin of the South American frog *Phyllomedusa sauvagei* contain, in addition to various other active peptides, dermorphin, a heptapeptide endowed with an exceptionally intense and long-lasting opioid activity. The structure of the new peptide presents the unique feature of having a D-amino acid residue in the molecule

Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2 Dermorphin
Tyr-D-Ala-Phe-Gly-Tyr-Hyp-Ser-NH_2 Hyp^6-dermorphin

The same extracts and, in greater abundance, extracts of the skin of the related species *P. rhodei* contain, in addition to dermorphin, the analogue Hyp^6-dermorphin (Hyp = hydroxyproline), as well as the corresponding deamidated peptides. The structures proposed for the natural peptides (Montecucchi, de Castiglione, Piani, Gozzini & Erspamer, 1981; Montecucchi, de Castiglione & Erspamer, 1981) have been confirmed by synthesis (de Castiglione, Faoro, Perseo & Piani, 1981).

In the present work some peripheral and central actions of dermorphin and a limited number of its natural and synthetic analogues are described and compared with those of Met-enkephalin, Leu-enkephalin, β-endorphin and morphine.

Methods

Opioid activity of dermorphin and other opiate-like compounds on peripheral receptor sites was estimated on the guinea-pig ileum longitudinal muscle myenteric plexus preparation and the mouse vas deferens preparation; central opioid activity was assessed by analgesia and catalepsy tests in mice and rats.

The guinea-pig ileum was prepared as described by Gyang & Kosterlitz (1966) and mounted in an organ bath of 15 ml capacity. The tissues were bathed in the modified Krebs solution (mM) described by Kosterlitz, Lydon & Watt (1970): NaCl 118, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 1.19, $MgSO_4$ 0.12, $NaHCO_3$ 25, glucose 11, containing hexamethonium chloride (0.069 mM) and mepyramine maleate (0.125 μM),

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gassed with 95% O_2 and 5% CO_2. The bath temperature was 36°C. The twitch-like contractions of the longitudinal muscle were recorded isometrically by a strain gauge transducer (DY 1, Basile, Milan) and displayed on a recording microdynamometer (Basile, Milan). The intramural nerves were stimulated with rectilinear pulses of 0.5 ms duration, given at intervals of 10 s, using supramaximal voltage. Usually, the ileum was exposed to opioid peptides for 3 to 5 min, until inhibition was maximal.

The mouse vas deferens was prepared as described by Hughes, Kosterlitz & Leslie (1975). One or, less frequently, two pairs of vasa were mounted in an organ bath of 8 ml capacity. Longitudinal contractions were recorded as described for the guinea-pig ileum. The tissues were bathed in modified Krebs solution (mM): NaCl 118, KCl 4.75, $CaCl_2$ 2.54, $KHPO_4$ 0.93, $NaHCO_3$ 25, glucose 11, containing ascorbic acid (0.1 mM) and sodium edetate (0.027 mM), gassed with 95% O_2 and 5% CO_2. The bath temperature was 36°C. The intramural nerves were stimulated with trains of rectilinear pulses of 1 ms duration. Trains were given at intervals of 15–20 s and consisted of 6–7 stimuli with intervals of 100 ms (Kosterlitz, personal communication). Pulses passed between a platinum point source at the bottom and a vertical ring (5 mm high and 10 mm wide) of platinum foil fixed at the top of the organ bath.

The analgesic effect of dermorphin was assayed in mice by the hot plate test (Ankier, 1974). Dermorphin, dissolved in 0.1 ml of 0.9% w/v NaCl solution (saline), or saline alone (controls) was injected into the tail vein in groups of 10 male IRC mice, weighing 25–30 g. The extent of analgesia was expressed as percentage of antinociceptive effect, as described by Harris & Pierson (1964). With a two fold increase in latency of reaction time as a quantal index of inhibition, the median antinociceptive dose (ED_{50}) and 95% confidence limits were calculated according to the method of Litchfield & Wilcoxon (1949). Reaction times of the order of 5 ± 2 s were observed in control mice.

The analgesic effect of dermorphin in the rat was assayed both by the hot plate and the tail-flick test. Male Wistar rats weighing 240–260 g were used. Dermorphin and related compounds were injected intracerebroventricularly, as described by Chermat & Simon (1975), in a volume of 10 μl saline. In the tail-flick test (D'Amour & Smith, 1941), the latency of withdrawal of tail exposed to radiant heat was measured automatically with an accuracy of 0.1 s. Groups of 10 animals were used in the single experiments. Reaction times to radiant heat and to hot plate were measured 30 and 15 min before the injection, and then every 15 min during the first post-injection hour, and every 30 min during the next 3 h. ED_{50} and 95% confidence limits were calculated as described for mice.

In chronic infusion studies for tolerance and physical dependence, 50 rats were anaesthetized with sodium pentobarbitone (50 mg/kg, i.v.) and an L-shaped plastic cannula (Yeda plastic cannulae, Linca Ltd., Israel) was then implanted into the lateral ventricle of each rat and secured to the skull with dental cement. An osmotic minipump (Alzet, Alza Corp., Palo Alto, Cal.) was used to deliver drugs to the brain. The minipump was prefilled with the drug solution and inserted subcutaneously between the scapulae. A 21 gauge steel tube protruding from the minipump was then coupled to the brain cannula with plastic tubes. To avoid dislodgement of the pump by the animal, the scalp wound was closed with sutures so that the entire infusion unit was enclosed below the skin. Analgesia was measured at intervals by the tail-flick test.

After 108 h infusion the animals were placed in glass jars and challenged with naloxone, 30 μmol/kg, s.c. 'Wet dog shakes' and leaping attempts to escape from the glass jar were counted over a 15 min period. If a rat made two or more escape attempts from the jar and three or more 'wet dog shakes' within 15 min after naloxone injection, it was considered to have undergone precipitated withdrawal.

To detect signs of catalepsy, animals were put in an almost erect position with their forepaws placed over the upper rung of an inverted toy stool. Cataleptic animals retained this posture for several seconds or even for a few minutes without struggling.

Table 1 Effects of dermorphin and other opiate-like compounds in two bioassay systems

	Guinea-pig ileum IC_{50} (nM)	*Mouse vas deferens* IC_{50} (nM)	*Potency ratio mouse vas deferens: guinea-pig ileum*
Dermorphin	3.30 ± 0.22 (32)	29 ± 3.35 (12)	0.11
Leu-enkephalin	970 ± 150 (8)	29.2 ± 3.16 (8)	33
Met-enkephalin	190 ± 30 (16)	26.8 ± 2.39 (7)	7
β-Endorphin	50,70 (2)	—	—
Morphine	150 ± 18.5 (6)	1215 ± 115 (4)	0.12

Mean values ± s.e. mean. Number of experiments in parentheses. —, not determined.

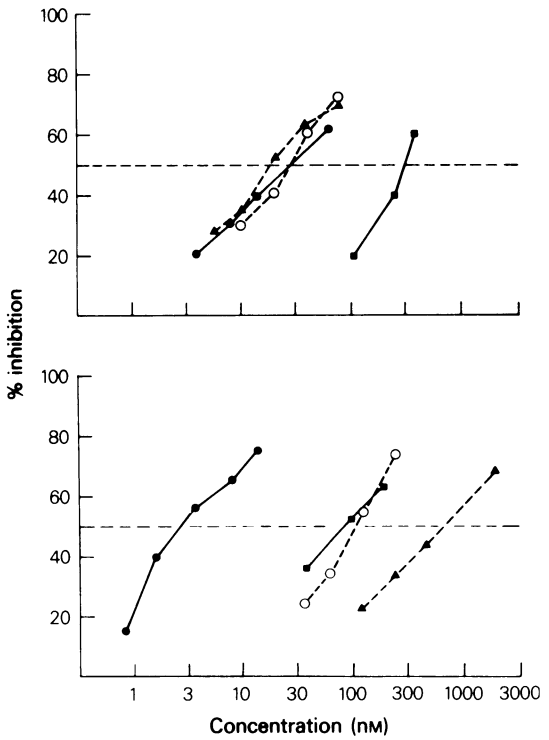

Figure 1 Typical dose-response relationship for dermorphin (●), morphine (■), Met-enkephalin (○) and Leu-enkephalin (▲) in a single preparation each of mouse vas deferens (upper) and guinea-pig ileum (lower). Broken horizontal line represents 50% twitch depression.

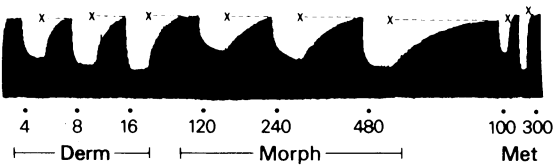

Figure 2 Guinea-pig ileum preparation. The effect of graded concentrations (nM) of dermorphin (Derm), morphine (Morph) and Met-enkephalin (Met), and duration of recovery, upon washing (×) with fresh bathing solution. Time marks, 5 min. Recovery was most rapid for Met-enkephalin, intermediate for dermorphin and slowest for morphine.

Peptides and drugs

Natural and synthetic dermorphin and Hyp^6-dermorphin, natural deamidated dermorphins, synthetic L-Ala^2-dermorphin and dermorphin fragments were prepared at the Farmitalia Carlo Erba Research Laboratories, Milan. Met-enkephalin and Leu-enkephalin were purchase from Serva, Heidelberg; β-endorphin from Beckman, Palo Alto, Cal.; naloxone hydrochloride (Narcan) from Endo Lab., New York, N.Y., morphine sulphate from Farmitalia Carlo Erba, Milan; pentobarbitone from Abbott, Chicago, Ill.

Results

Guinea-pig ileum preparation

The potency of dermorphin in the guinea-pig ileum preparation was, on the average, 57 times that of Met-enkephalin, 294 times that of Leu-enkephalin, 18 times that of β-endorphin, and 39 times that of morphine. The concentrations for 50% twitch inhibition (IC_{50}) are shown in Table 1. A typical dose-response relationship for dermorphin and other agonists in a single preparation is shown in Figures 1 and 2, where it may be seen that dermorphin produced an excellent dose-related response, with no sign of tachyphylaxis. Response to dermorphin was somewhat slower in onset than that to the enkephalins, but somewhat faster than that to morphine. Duration of effect, as inferred from duration of maximum twitch depression produced by equiactive doses, was again shortest for the enkephalins, intermediate for dermorphin, and longest for morphine.

An example of reversal of twitch depression upon washing the ileum preparation by overflow in shown in Figure 2. It may be seen that recovery for dermorphin took 5 min for a 50% twitch depression and 7 min for a 65% twitch depression, whereas for morphine, recovery took 8 min for a 40% twitch depression and 20 min for a 64% twitch depression. In the case of Met-enkephalin recovery took less than 1 min for a 70% twitch depression. Thus, rate of reversal for dermorphin was intermediate between that for morphine and that for Met-enkephalin.

Addition to the bath, without washing, of 2–3 successive, equal doses of dermorphin, at intervals of 3–5 min, often caused the same final twitch depression as that obtained with a single dose summing the divided doses. The same was observed for morphine. Testing a standard dose of morphine in the presence of a partially (30–40%) inhibiting concentration of dermorphin, as described by Kosterlitz & Watt (1968), produced simple additive effects, indicating that dermorphin had no appreciable antagonistic properties to morphine.

Naloxone was a potent antagonist of dermorphin. The ratios of IC_{50} values in the presence and absence of 10 nM naloxone were (number of experiments in parentheses) similar for dermorphin 4.5 ± 0.27 (6), Met-enkephalin 3.8 ± 0.31 (4), Leu-enkephalin 3.75 ± 0.29 (4) and morphine 3.38 ± 0.24 (4).

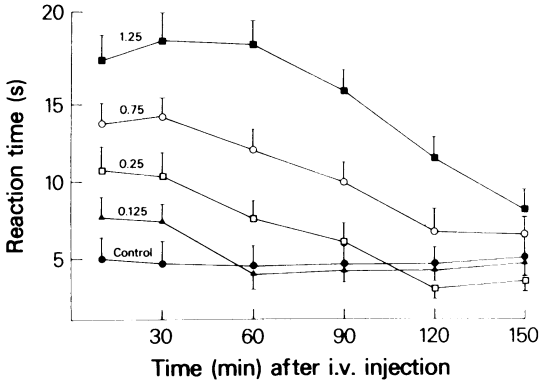

Figure 3 Time-response curves for analgesic activity in hot plate test of different doses of dermorphin (μmol/kg), given by intravenous injection in groups of ten mice each. Vertical bars show s.e. mean.

Mouse vas deferens preparation

Dermorphin in this preparation was as active as Leu-enkephalin and Met-enkephalin and approximately 40 times more potent than morphine. IC_{50} values are shown in Table 1. Again twitch depression produced by dermorphin showed an excellent dose-response relationship, and was prompt both in onset and in disappearance, upon washing. Spontaneous recovery was less pronounced for dermorphin than for the enkephalins and recovery upon washing slightly retarded in comparison to the enkephalins, but more rapid than with morphine.

Naloxone was considerably more potent in blocking dermorphin than in blocking the enkephalins. In fact, the ratios of IC_{50} values in the presence and absence of a fixed naloxone concentration (20 nM) were (number of experiments in parentheses) 5.7 ± 0.66 (5) for dermorphin, 1.6 ± 0.05 (4) for Met-enkephalin and 1.8 ± 0.07 (4) for Leu-enkephalin.

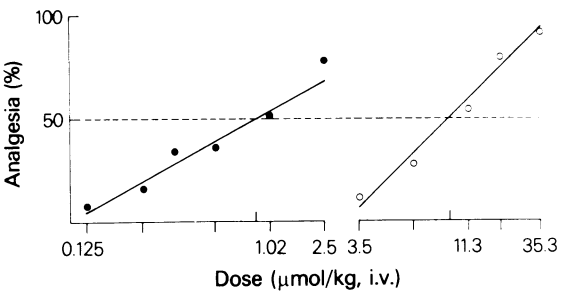

Figure 4 Mice, hot plate test. Comparison of antinociceptive effects of dermorphin (●), and morphine (○), given by intravenous injection, in groups of ten animals each. Doses in μmol/kg. The ED_{50} of dermorphin was 1.02 (0.67–1.55), that of morphine 11.3 (7.4–20.8).

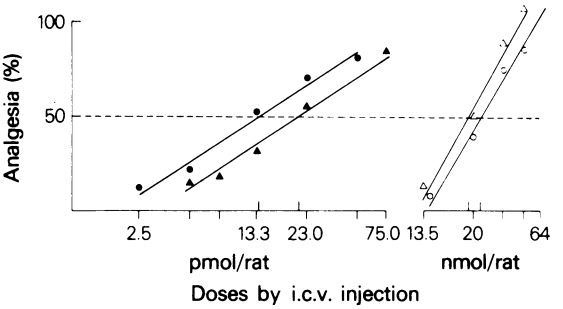

Figure 5 Comparison of antinociceptive effects of different doses of dermorphin (● ▲) and morphine (○ △) given in rats (groups of ten animals each) by intracerebroventricular (i.c.v.) injection. (● ○) hot plate test; (▲ △) tail-flick test. ED_{50} for dermorphin was 13.3 (8.3–22.9) pmol/rat in the hot plate test and 23 (15.3–40) pmol/rat in the tail-flick test; ED_{50} for morphine was 28.3 (18.4–46.9) nmol/rat in the hot plate test and 17.8 (10.9–24) nmol/rat in the tail-flick test.

Analgesia in mice

Dermorphin given to mice at intravenous doses of 0.125, 0.25, 0.75 and 1.25 μmol/kg, produced a dose-related inhibition of the hot plate response (Figure 3). Threshold was approximately 0.125 μmol/kg. Analgesia lasted 60–150 min, depending on the dose, and appeared to be of shorter duration than analgesia in rats following intracerebroventricular injection of the peptide. Pretreatment of mice with naloxone (3 μmol/kg, s.c.) 5 min before injection of 1.25 μmol/kg dermorphin completely abolished the analgesic effect of the peptide.

The percentage of mice showing analgesia after intravenous injection of graded doses of dermorphin and morphine and their ED_{50} values with 95% confidence limits are shown in Figure 4. It may be seen that the ED_{50} values of dermorphin (1.02 μmol/kg, 0.67–1.55) were about 11 times lower than those of morphine (11.3 μmol/kg, 7.4–20.8).

Of the three fragments of dermorphin studied, the *N*-terminal pentapeptide showed the highest activity, reaching 50% that of dermorphin, while the *N*-terminal tetrapeptide appeared to represent the shortest sequence necessary for analgesic activity in mice.

Analgesia and catalepsy in rats

Dermorphin displayed very potent analgesic activity in rats, when given by intracerebroventricular injection. The ED_{50} of the peptide in the tail-flick test was 23 pmol/rat (15.3–40), the ED_{50} of morphine was 752 times higher (17.3 nmol/rat, 10.9–24). In the hot plate test (Figure 5) dermorphin was still more potent, both absolutely and in comparison to morphine. In fact,

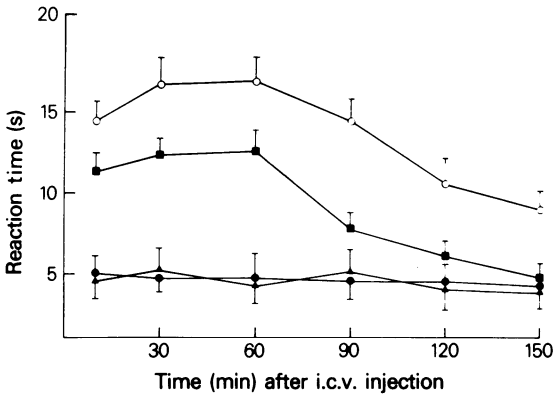

Figure 6 Time-response curves for analgesic activity of a single dose of dermorphin (60 pmol/rat) administered intracerebroventricularly (i.c.v.) in groups of 10 animals each. Responses obtained in the hot plate test (○) and in the tail-flick test (■). Controls: hot plate test (●) and tail-flick test (▲). Vertical bars show s.e. mean.

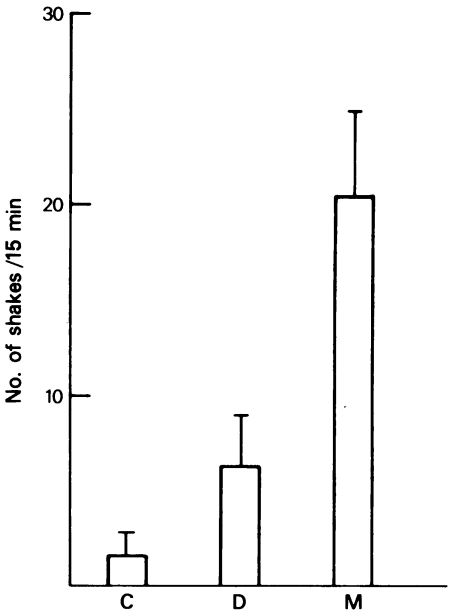

Figure 7 Naloxone-precipitated shaking behaviour in rats infused intracerebroventricularly with saline (C), dermorphin (D) and morphine (M) (see text for procedures). Each column represents the mean value obtained from 10 animals; vertical bars show s.e. mean.

the ED_{50} for dermorphin was 13.3 pmol/rat (8.3–22.9) and for morphine 28.3 nmol/rat (18.4–46.9). Thus, dermorphin was 2170 times more potent than morphine.

Following intracerebroventricular injection of 60 pmol dermorphin/rat, analgesia lasted approximately 90 min when determined in the tail-flick test, and more than 150 min when determined in the hot plate test (Figure 6).

Doses of dermorphin higher than 130 pmol/rat produced a cataleptic response, lasting 180–240 min. This was accompanied by tachypnoea, mydriasis, hyperexcitability and rigid tail. The rat, when lightly touched, made rapid brisk movements.

Table 2 shows the analgesic and cataleptic response of the rat to some dermorphin analogues and N-fragments.

Development of tolerance and physical dependence

Dermorphin was infused intracerebroventricularly into the rat at a rate of 62 pmol/h for 5 days. Checking the rats with the tail-flick test, analgesia was evident in 95% of animals after 4 h of infusion, in 82% after 24 h, and in 65% after 4 days. Thus the appearance of tolerance was obvious, but in its onset and development it was considerably slower than that observed in rats given equianalgesic doses of morphine (17.6 nmol/h). In fact, analgesia was demonstrated in only 10% of morphine-treated rats after 4 days of continuous infusion.

On the 5th day of infusion, when the osmotic pump was no longer functional, all the rats underwent abrupt withdrawal symptoms when injected with 30 μmol/kg naloxone subcutaneously. The syndrome was characterized in morphine-treated rats by frequent shaking, teeth chattering, yawning, sneezing and occasional licking of the penis. In dermorphin-treated animals the syndrome was less pronounced, and only shaking was observed. Shaking behaviour in morphine and dermorphin-infused animals is shown in Figure 7. Morphinized rats showed more than 20 shakes in 15 min, while dermorphin-treated animals never exceeded 10 shakes, with a mean of 6 shakes per 15 min.

Relative potency of natural dermorphins and synthetic dermorphin fragments

The relative potency of some natural and synthetic dermorphins or dermorphin fragments is shown in Table 2. It should be noted that synthetic dermorphin and Hyp^6-dermorphin displayed 80–115% of the activity of the corresponding natural peptides.

Tabulated data show that the shortest amino acid sequence required for the appearance of opioid activity was represented by the *N*-terminal tetrapeptide, with the presence in the dermorphin molecule of the D-Ala residue being of crucial importance. A clear-cut dissociation between analgesia and catalepsy was evident in the *N*-terminal tetra- and pentapeptides.

Table 2 Relative potency of natural dermorphins and synthetic dermorphin fragments in four *in vitro* and *in vivo* test preparations (dermorphin = 100)

	Guinea-pig ileum	*Mouse vas deferens*	*Rat hot plate test*[1]	*Rat catalepsy*[1]
Dermorphin	100	100	100	100
L-Ala^2-dermorphin	<0.03	<0.05	<0.02	<0.2
Hyp^6-dermorphin	80–90	85–90	90–110	100
Tyr-D-Ala-Phe-Gly-Tyr-Pro-NH_2	50–60	40–45	25	1
Tyr-D-Ala-Phe-Gly-Tyr-NH_2	50–70	70–80	27	0.5–1
Tyr-D-Ala-Phe-Gly-NH_2	4–6	6	25	<0.5
Tyr-D-Ala-Phe	<0.03	<0.05	<0.2	<0.2
Tyr-Pro-Ser-NH_2	<0.03	<0.03	<0.02	<0.2

[1]Peptides injected into the lateral ventricle

Discussion

Present research demonstrates that dermorphin is an extraordinarily potent opioid peptide and considering the entire spectrum of its biological activity, probably the most potent of the natural peptides. In the intensity of its effects on the opiate receptors of the guinea-pig intestine and mouse vas deferens, dermorphin was slightly less active than (1–13)-dynorphin (Goldstein, Tachibana, Lowney, Hunkapiller & Hood, 1979), but in its analgesic effects dermorphin was superior to all other natural opioid peptides and also most, if not all, the synthetic opioid peptides.

In mice an analgesic effect was obtained even by intravenous injection of dermorphin, the peptide being 11 times more potent than morphine. But the most striking opioid effects of dermorphin on the CNS were displayed following intracerebroventricular injections in rats. Dermorphin was exceptionally potent both in the tail-flick test (ED_{50} = 23 pmol/rat = 18 ng/rat) and, even more, in the hot plate test (ED_{50} = 13 pmol/rat = 10 ng/rat). In our experiments dermorphin was 750 and 2170 times more potent than morphine, and from data reported in the literature the peptide may be calculated to be at least 1000 times more potent not only than the virtually inactive natural enkephalins but also than dynorphin (Goldstein *et al.*, 1979), and at least 30–50 times more potent than β-endorphin (Rossier & Bloom, 1979).

Dermorphin is characterized by a long duration of action, both peripherally and centrally, which appears to be due mainly to its resistance to enzymatic attack on account of the presence of the D-Ala^2 residue and, subordinately, of the Pro^6 residue in the molecule. The ligand-receptor dissociation rate is probably of minor importance in the duration of the effects of dermorphin.

All the central and peripheral effects of dermorphin considered in this study were readily antagonized by naloxone, given prior to or after the peptide. In the guinea-pig ileum preparation the antagonist was equipotent in blocking dermorphin, the enkephalins and morphine but in the mouse vas deferens preparation, naloxone was 3–4 times more potent in blocking dermorphin than in blocking the enkephalins.

The appearance of moderate tolerance to dermorphin was seen in rats given an intracerebroventricular infusion of the peptide for 5 days. However, onset and development of this phenomenon were much slower in dermorphin-rats than in rats given equianalgesic doses of morphine. Similarly, withdrawal symptoms, precipitated by naloxone treatment were considerably less severe in dermorphin-infused rats than in morphine-infused rats. This part of our study, however, should be considered only preliminary, further experiments in rats and other animal species being necessary.

More than 90 dermorphin-like analogues have so far been prepared by synthesis at the Farmitalia Carlo Erba Research Laboratories, Milan. Their pharmacological properties and relative potencies will be described in detail elsewhere. However, some conclusions may be drawn from the few data reported in this paper: (1) Hyp^6-dermorphin possesses approximately the same activity as dermorphin; (2) the D-Ala^2 residue is of crucial importance for opioid activity, in fact, L-Ala^2-dermorphin is virtually inactive; (c) the minimum length of the dermorphin molecule required for full opioid activity is represented by the *N*-terminal tetrapeptide.

Apart from the obvious interest related to the discovery of a new class of amphibian skin peptides endowed with exceptionally intense opioid activity, studies on dermorphins revealed two important observations of general significance.

The first observation is the occurrence, in the dermorphin molecule, of a D-Ala residue which is crucial, as already pointed out, for activity. To our knowledge, this is the first example of the presence, in

vertebrate tissues, of a D-amino acid residue in a peptide molecule. The possibility of a racemization of L-Ala^2-dermorphin pre-existing in fresh skin during extraction and purification procedures has been discussed and excluded by Montecucchi *et al.* (1981). The second observation is that once again amphibian skin has proved to be a source of potent peptides, generally having their counterpart in mammalian tissues, especially brain and intestine.

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