Potentiation of dermorphin-induced antinociception by peptidase inhibitors

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We have investigated the effect of amastatin, an aminopeptidase inhibitor, and captopril, an angiotensin converting enzyme inhibitor, on the antinociceptive activity induced by intracerebroventricular administration of dermorphin, a heptapeptide. In addition, the potency of dermorphin was compared with that of one of its metabolites, N-terminal tetrapeptide (Tyr-D-Ala-Phe-Gly), by using the tail pressure test. The antinociceptive activity induced by dermorphin was not potentiated by simultaneous administration of amastatin or captopril. However, there was potentiation when dermorphin was combined with both peptidase inhibitors. Moreover, the N-terminal tetrapeptide was 84 times less potent than its parent heptapeptide when administered intracerebroventricularly. The results suggest that the cleavage of Tyr^1-D-Ala^2 and Gly^4-Tyr^5 bonds by brain endopeptidase modulates dermorphin-induced antinociceptive activity.

Dermorphin, extracted from the skin of frogs of genus Phyllomedusa (Erspamer & Melchiorri 1980), is a heptapeptide (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH$_2$) with exceptionally potent and long-lasting peripheral and central activity. In the guinea-pig isolated ileum, dermorphin is much more potent than met-enkephalin, leu-enkephalin and morphine (Kisara et al 1986). Dermorphin with opioid-like activity was shown to display a potent and long-lasting antinociception by intravenous and intracerebroventricular (i.c.v.) administration (Broccardo et al 1981). Dermorphin is unique among the naturally occurring peptides in the respect that it is the first example of a peptide of non-bacterial origin containing a D-amino acid residue in its sequence. Structure-activity relation studies with dermorphin have shown that the N-terminal tetrapeptide is the minimal requirement for potent opioid-like antinociceptive activity and biological effects, but this fragment is less potent than the parent heptapeptide (De Castiglione et al 1981). The main degraded products of dermorphin are thought to be an N-terminal tripeptide in peripheral organs and an N-terminal tetrapeptide in the brain (Negri & Improta 1982). Based on this report, we directed our interest to the role of endopeptidase activity in modulating the dermorphin response. To this end, amastatin, an aminopeptidase inhibitor (Aoyagi et al 1978), and captopril, an angiotensin converting enzyme inhibitor (Rubin et al 1978) were tested for their ability to potentiate the antinociceptive activity induced

by dermorphin. In addition, the parent heptapeptide potency was compared with its N-terminal tetrapeptide.

Materials and method

Male ddY-mice, 22–24 g, were housed at 20–24 °C in an automatically lit room (lights on at 0900h and off at 2100h) and had free access to food and water. All drugs for i.c.v. administration were freshly prepared in Ringer solution. The technique for i.c.v. administration was as described by Orikasa et al (1980). The drugs used for administration were captopril (Sankyo) and amastatin (Peptide Research Foundation). The synthesis of dermorphin has been partially discussed elsewhere (Sasaki et al 1985).

To assess the antinociceptive activity, the tail pressure test, which was slightly modified from the original method (Green et al 1951), was used. The base of the tail was pressed and the level of pressure in mmHg (20 mmHg s^{-1}) that evoked biting or struggling behaviour was noted. Groups of mice (n = 10) were selected for each experiment, no animal being used more than once. Only mice responding behaviourally to mechanical nociceptive stimulation (80–100 mmHg) were selected. A value of 200 mmHg was used as the cut-off pressure. The antinociceptive activity for each mouse was calculated according to the following formula: % of antinociception = $(P_2 - P_1/200 - P_1) \times 100$, where P_1 is the responsive pressure before drug administration (mmHg) and P_2 is the responsive pressure after drug administration (mmHg). The data were expressed as mean % of antinociceptive response ± s.e. At 5, 10, 15, 30, 45, 60, 90 and 120 min following administration, tail pressure thresholds were redetermined. Statistical significance of the data was estimated by a mixed several factor ANOVA with Tukey's test (May 1952). ED50 and 95% confidence limits were calculated by the method of Litchfield & Wilcoxon (1949).

Results

The co-administration of an aminopeptidase inhibitor, amastatin, and an angiotensin converting enzyme inhibitor, captopril, brought about a significant enhancement of antinociceptive activity induced by i.c.v. administration of dermorphin in mice. Captopril or amastatin alone were without effect in producing antinociceptive activity. Table 1 shows the time course of the antinociceptive response of dermorphin (7·6

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Table 1. The time-course effects of dermorphin when co-administered with captopril and/or amastatin in mice.

	5 min	10 min	15 min	30 min	45 min	60 min	90 min	120 min
Ringer control	−0·2 ± 1·3	0·8 ± 2·0	0·0 ± 2·6	1·1 ± 0·9	−2·6 ± 1·5	0·1 ± 1·5	−0·6 ± 1·7	0·3 ± 1·8
Dermorphin	29·0 ± 6·0	48·8** ± 8·1	38·0** ± 4·4	32·4 ± 5·4	21·4 ± 5·9	19·0 ± 4·0	18·3 ± 6·5	5·8 ± 2·9
Dermorphin + captopril (50 µg)	51·1* ± 9·6	63·9** ± 7·2	52·6** ± 6·4	46·9* ± 11·9	38·2* ± 8·9	23·0 ± 5·3	18·8 ± 3·8	5·9 ± 2·6
Dermorphin + amastatin (10 µg)	37·9 ± 8·4	36·7** ± 5·4	45·2** ± 6·7	57·0** ± 6·7	47·9* ± 5·4	29·4 ± 6·8	26·5 ± 6·4	18·2 ± 4·3
Dermorphin + captopril + amastatin	70·8*†† ± 9·8	91·9*†† ± 3·0	85·9*†† ± 6·9	84·0*†† ± 7·5	79·3**†† ± 7·9	78·1*†† ± 9·4	51·9**† ± 10·2	45·6*†† ± 7·5

Each value was expressed as % of MPE.
A dose of 7·6 pmol/mouse of dermorphin was administered i.c.v.
* $P < 0.05$ and ** $P < 0.01$ when compared with Ringer control.
† $P < 0.05$ and †† $P < 0.01$ when compared with dermorphin.

Table 2. Effect of i.c.v. administration of captopril and/or amastatin on dermorphin-induced antinociceptive activity in mice.

Drugs	ED50 (pmol/mouse)
Morphine	1200·0 (700·0–2200·0)
H-Tyr-D-Ala-Phe-Gly-OH	510·0 (351·7–759·5)
Dermorphin	6·9 (4·5–10·6)
Dermorphin + captopril (50 µg)	6·1 (3·8–9·7)
Dermorphin + amastatin (10 µg)	6·6 (4·4–10·0)
Dermorphin + captopril (50 µg) + amastatin (10 µg)	2·0 (1·2–3·3)

ED50 values were calculated from the values obtained at the time of peak effect. 95% confidence limits are given in parentheses.

pmol/mouse i.c.v.) in the absence and presence of the peptidase inhibitors. Dermorphin (7·5 pmol/mouse i.c.v.) exhibited a long-lasting activity which peaked at 10 min and fell away over 120 min as measured by the tail-pressure test. This was not significantly potentiated when dermorphin was combined with captopril 50 µg or amastatin 10 µg. But a more potent and long-lasting increase over that induced by dermorphin alone was achieved when captopril and amastatin were given together. The antinociceptive duration was also much longer than that of the parent heptapeptide alone; even at 120 min an antinociceptive activity of approximately 45% was obtained. Table 2 shows the ED50 of dermorphin in the absence and presence of inhibitors and a metabolite of dermorphin, tetrapeptide.

The N-terminal tetrapeptide was 84 times less potent than dermorphin and its action peaked at 10 min and

disappeared much more rapidly than dermorphin (Table 1). The ED50 value of tetrapeptide was 510·0 (351·7–739·5) pmol (Table 2).

Discussion

The chemical structure of dermorphin presents the unique feature of having a D-Ala residue incorporated in its N-terminal sequence. The L-Ala dermorphin is virtually inactive. Likewise in enkephalin analogues, the substitution of Gly[2] with D-Ala[2] results in peptides with exceptionally increased activity as represented by [D-Ala[2], Met[5]]enkephalinamide (Pert et al 1976) or FK-33824 Tyr-D-Ala-Gly-MePhe-Met(O)ol (Roemer et al 1977). These two derivatives having the Tyr-D-Ala unit were more slowly degraded than enkephalin, by release of Tyr as the first amino acid, upon incubation with mouse brain extracts (Huguenin & Maurer 1980). A fragment of the dermorphin analogue, Tyr-D-Arg-Phe-Gly was slowly cleaved by aminopeptidase M, compared with the tetrapeptide having the L-amino acid in the second position (Sasaki et al 1985). By analogy with degradation of enkephalin analogues and the dermorphin fragment, the release of Tyr might be the determinant of dermorphin catabolism.

On the other hand, dermorphin is degraded in peripheral organs of rat to produce the N-terminal tripeptide as a main degradation product, while the parent heptapeptide is slowly degraded to the N-terminal tetrapeptide by brain endopeptidase (Negri & Improta 1982). In the present work, we found the tetrapeptide to be approximately 84 times less potent than the parent peptide when administered i.c.v. This

indicates that the cleavage of the Gly^4-Tyr^5 bond may decrease the antinociceptive activity of dermorphin.

We have recently demonstrated that resistance to the cleavage of Tyr^1-D-Ala^2 was strengthened by amastatin and that captopril inhibited the cleavage of Gly^4-Tyr^5 and Pro^6-Ser^7 bonds in the dermorphin molecule by endopeptidase. Furthermore, both peptidase inhibitors prevented three sites of peptide bonds upon incubation with rat brain extracts (unpublished data).

In the present experiment, dermorphin with captopril or amastatin, produced an antinociceptive effect which lasted longer than that produced by dermorphin alone when compared with Ringer control, but there was no significant increase in potency. The finding that dermorphin-induced antinociceptive activity was dramatically enhanced when the heptapeptide was concurrently administered with captopril and amastatin means, at least, that either cleavage of the Tyr^1-D-Ala^2 or Gly^4-Tyr^5 bonds may decrease dermorphin-induced antinociceptive activity.

Our present findings suggest that one of the catabolic pathways modulating dermorphin-induced antinociceptive activity by means of endopeptidase, is the cleavage of Tyr^1-D-Ala^2 bond. The other produces the N-terminal tetrapeptide which is less potent than its parent heptapeptide.

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The effect of ascorbate on the acetylcholine release from guinea-pig ileal myenteric plexus

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The effect of ascorbate on the release of acetylcholine from the longitudinal muscle-myenteric plexus preparation of the guinea-pig isolated ileum has been investigated using a bioassay and an isotopic technique. Ascorbate at 5 mM increased the spontaneous output of acetylcholine and enhanced DMPP-induced output of acetylcholine, while iso-ascorbate, 5 mM, did not. Ascorbate did not influence either the spontaneous or the DMPP-induced release of acetylcholine from synaptosomes of the ileal myenteric plexus. These results suggest that ascorbate promotes acetylcholine release from intramural cholinergic nerves.

We have previously shown that ascorbate elicits a contractile response in the guinea-pig isolated ileum and have suggested that this response is due to a release of acetylcholine(ACh) from the myenteric plexus (Terada

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et al 1980). We have also demonstrated that ascorbate augments the contraction induced by 1,1-dimethyl-4-phenylpiperazinium(DMPP), a nicotinic agonist, in the ileal longitudinal muscle of guinea-pig, and proposed that it might promote the ACh-releasing action of DMPP at the ganglion cells in the myenteric plexus (Hayashi et al 1983). Kuo et al (1979) and Kuo & Yoshida (1980) have reported that ascorbate causes a release of ACh from isolated synaptic vesicles of brain in rat, guinea-pig and rabbit. Furthermore, Pinchasi et al (1979) demonstrated that ascorbate induces Ca^{2+}-dependent release of ACh from synaptic vesicles isolated from *Torpedo* spp. In the present study, we report on the effect of ascorbate on ACh release from the longitudinal muscle strip of guinea-pig isolated ileum and its influence on ACh release from the