0022-3565/98/2862-0863$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 286, No. 2
Printed in U.S.A.

JPET 286:863–869, 1998

Protection against Dynorphin-(1–8) Hydrolysis in Membrane Preparations by the Combination of Amastatin, Captopril and Phosphoramidon[1]

TOYOKAZU HIRANUMA, KEN KITAMURA, TAKAO TANIGUCHI, MASAYUKI KANAI, YOSHIHARU ARAI, KAYOKO IWAO and TETSUO OKA

Department of Pharmacology, School of Medicine, Tokai University, Isehara 259-11, Japan

Accepted for publication April 10, 1998 This paper is available online at http://www.jpet.org

ABSTRACT

The amounts of dynorphin-(1–8) [dyn-(1–8)] and its seven hydrolysis products, Y, YG, YGG, YGGF, YGGFL, YGGFLR and YGGFLRR, were estimated after incubating dyn-(1–8) with a membrane fraction from either guinea-pig ileum or striatum for various times at 37°C. The major hydrolysis products during the initial 5-min incubation were YGGFLR and Y, which indicates that dipeptidyl carboxypeptidase and aminopeptidase activities were mainly involved in the hydrolysis. After 60 min of incubation, dyn-(1–8) was completely hydrolyzed in both membrane preparations. When the ileal and the striatal preparations were incubated for 60 min in the presence of both captopril, a dipeptidyl carboxypeptidase inhibitor, and amastatin, an aminopeptidase inhibitor, 63.8 and 49.3% of dyn-(1–8), respectively, were hydrolyzed. The YGG fragment was the major hydrolysis product in both preparations. When the ileal and the striatal membrane fractions were incubated with dyn-(1–8) in the presence of three peptidase inhibitors, captopril, amastatin and phosphoramidon (an inhibitor of endopeptidase-24.11), approximately 95% of the opioid octapeptide remained intact in both cases. This shows that dyn-(1–8) was almost exclusively hydrolyzed by three enzymes, amastatin-sensitive aminopeptidase, captopril-sensitive dipeptidyl carboxypeptidase I and phosphoramidon-sensitive endopeptidase-24.11, in both ileal and striatal membranes. Additionally, the Ke (equilibrium dissociation constant) values of selective antagonists against dyn-(1–8) and its initial main hydrolysis product YGGFLR in two isolated preparations pretreated with the three peptidase inhibitors indicate that the latter acts on *mu* receptors in guinea pig ileum but *delta* receptors in mouse vas deferens and the former acts on *kappa* receptors in both preparations. It is indicated, therefore, that in the absence of peptidase inhibitors endogenously released dyn-(1–8) acts either through dyn-(1–8) itself on *kappa* receptors or through YGGFLR on *mu* or *delta* receptors depending on both the three peptidase activities and the three receptor type densities at the target synaptic membrane.

Three distinct enzymes, AsA, PsE and CsD, have been shown to play important roles in the inactivation of exogenously added opioid peptides in isolated preparations: [Met^5]-enkephalin (met-enk) in guinea pig ileum (Aoki *et al.*, 1984), mouse vas deferens (Aoki *et al.*, 1986) and rat vas deferens (Cui *et al.*, 1986); dynorphin-(1–8) [dyn-(1–8)] in guinea pig ileum, mouse vas deferens and rabbit vas deferens (Numata *et al.*, 1988) and met-enk-Arg^6-Gly^7-Leu^8 (met-enk-RGL) in guinea pig ileum (Hiranuma *et al.*, 1997). The close proximities of these enzymes to the opioid receptors in guinea pig ileum (Aoki *et al.*, 1984) and mouse vas deferens (Aoki *et al.*, 1986) suggest that they function in terminating the physiological action of endogenous opioid peptides as well. Additionally, when ileal and striatal membrane fractions are incubated with met-enk (Hiranuma and Oka, 1986) or met-enk-RGL (Hiranuma *et al.*, 1997) for 60 min at 37°C in the presence of three PIs (amastatin, captopril and phosphoramidon), approximately 95% of both peptides remain intact. This shows that both met-enk and met-enk-RGL are almost exclusively hydrolyzed by the three enzymes, AsA, PsE and CsD, at least in these membrane preparations.

The enzymes involved in the hydrolysis of endogenous

Received for publication January 28, 1998.
[1] This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

ABBREVIATIONS: dyn-(1–8) and -(1–17), dynorphin-(1–8) and -(1–17); AsA, amastatin-sensitive aminopeptidase(s); PsE, phosphoramidon-sensitive endopeptidase-24.11 ("enkephalinase", EC 3.4.24.11); CsD, captopril-sensitive dipeptidyl carboxypeptidase I (angiotensin 1 converting enzyme, kininase II, EC 3.4.15.1); met-enk, [Met^5]-enkephalin; met-enk-RGL, [Met^5]-enkephalin-Arg^6-Gly^7-Leu^8; PI, peptidase inhibitor; DAMGO, [D-Ala^2, *N*-Me-Phe^4,Gly-ol]-enkephalin; DPDPE, [D-Pen^{2,5}]-enkephalin; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2; nor-BNI, nor-binaltorphimine HCl tetrahydrate; ICI-174,864, *N,N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (Aib = α-aminoisobutyric acid); HPLC-ECD, high-performance liquid chromatography combined with electrochemical detection; The standard one-letter, instead of three-letter, codes for amino acids were employed for the hydrolysis products of dyn-(1–8).

opioid peptides other than the two peptides met-enk and met-enk-RGL, however, have not been throughly investigated. In the case of dyn-(1–8), an endogenous opioid peptide that is derived from proenkephalin B (Kakidani *et al.*, 1982) and acts on *kappa* receptors (Corbett *et al.*, 1982; Oka and Negishi, 1982), its potency in guinea pig ileum, mouse vas deferens and rabbit vas deferens has been shown to be significantly increased by pretreatment of the preparations with the combination of either four PIs (Corbett *et al.*, 1982; McKnight *et al.*, 1983) or three PIs (Numata *et al.*, 1988). These results suggest the involvement of several peptidases in its inactivation. However, it was still unclear how to prevent completely the hydrolysis of dyn-(1–8). This made it impossible to determine the potency of dyn-(1–8) accurately, inasmuch as the determinations were made on partially degraded peptide. Therefore, we explored methods to protect dyn-(1–8) completely from degradative hydrolysis. Upon establishing a method that affords the peptide complete protection, we extended our study to determine the potency of dyn-(1–8) accurately and compared it with the potencies of representative opioid agonists.

Materials and Methods

Chemicals. Ketocyclazocine was a gift from Sterling-Winthrop Research Institute (Rensselaer, NY). Captopril was kindly provided by Sankyo Company (Tokyo, Japan). Other reagents were purchased from the following sources: dyn-(1–8), DAMGO, DPDPE and CTOP (Peninsula Laboratories, Inc., Belmont, CA); Y (tyrosine) [The standard one-letter, instead of three-letter, codes for amino acids were used for the hydrolysis products of dyn-(1–8)], YGGFL ([Leu^5]-enkephalin), dynorphin-(1–17) [dyn-(1–17)], amastatin and phosphoramidon (Peptide Institute, Inc., Minoh, Japan); YG, YGG, YGGF, YGGFLR and YGGFLRR (BACHEM Feinchemikalien AG, Bubendorf, Switzerland); morphine HCl (Sankyo Company) and nor-BNI and *N,N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (Aib = α-aminoisobutyric acid) (ICI-174864) (Research Biochemicals, Inc., Natick, MA). All chemicals, except for ketocyclazocine, were dissolved in glass-distilled water. Ketocyclazocine was dissolved in 0.5 N lactic acid at a concentration of 30 mM and then diluted with water. The stock solution for all peptides used was prepared at concentrations of 0.1 to 10 mM in siliconizing plastic tubes, maintained at −18°C and then diluted to the desired concentration just before use.

Preparation of membrane fractions. Male Hartley guinea pigs weighing 400 to 600 g were used for this study. The myenteric plexus-longitudinal muscle strip was prepared as previously described (Oka *et al.*, 1982). The striatum of the guinea pig brain was dissected by the method of Glowinski and Iversen (1966). Membrane fractions of the strips or striata were prepared as previously described (Hiranuma and Oka, 1986; Hiranuma *et al.*, 1997). They were suspended and adjusted with 50 mM Tris-HCl buffer, pH 7.4, to a concentration of 2 mg/ml of protein, yielding the sample used in the after experiment as the membrane fraction. Protein concentrations were measured by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Hydrolysis of dyn-(1–8). The sample of either the ileal or striatal membrane fraction (0.5 mg of protein) in 50 mM Tris-HCl buffer, pH 7.4, was incubated with dyn-(1–8) (5 nmol) in either the absence or the presence of the PIs for various times at 37°C in a total volume of 0.5 ml. The reaction was stopped by the addition of 0.5 ml of 10% trichloroacetic acid, followed by centrifugation at 3000 rpm for 15 min. A sample (10–20 μl) of the supernatant was then analyzed by HPLC-ECD to assess the product formation.

Separation and detection of dyn-(1–8) and its hydrolysis products by HPLC-ECD. The apparatus consisted of a PM-60 pump (Bioanalytical Systems, Inc., West Lafayette, IN), a μBondasphere 5-μm C18-100Å column (3.9 × 150 mm) (Waters/Nihon Millipore, Ltd., Tokyo, Japan) and an LC-4B electrochemical analyzer (Bioanalytical System, Inc.). The chromatographic mobile phase was 0.1 M phosphate buffer at pH 3.0 containing 2.5% acetonitrile for the Y, YG and YGG assays (fig. 1A); 12% acetonitrile for the YGGF, YGGFL, YGGFLR and YGGFLRR (fig. 1B); and 17% acetonitrole for the dyn-(1–8) (fig. 1C), at the flow rate of 0.8 ml/min. The ECD worked at an applied voltage of 1 V *vs.* the Ag/AgCl reference electrode. Under these conditions, Y, YG, YGG, YGGF, YGGFL, YGGFLR, YGGFLRR and dyn-(1–8) exhibited a retention time of approximately 3.58, 5.38, 4.77, 4.23, 19.6, 7.10, 4.71 and 4.88 min, respectively (fig. 1A, B and C), and the detection limit of each compound was approximately 2 pmol. The recovery of dyn-(1–8) and those of its hydrolysis products added to the heat-treated membrane fraction (100°C for 5 min) was each more than 95%. Therefore, the amount of each compound was estimated without correcting for its % recovery.

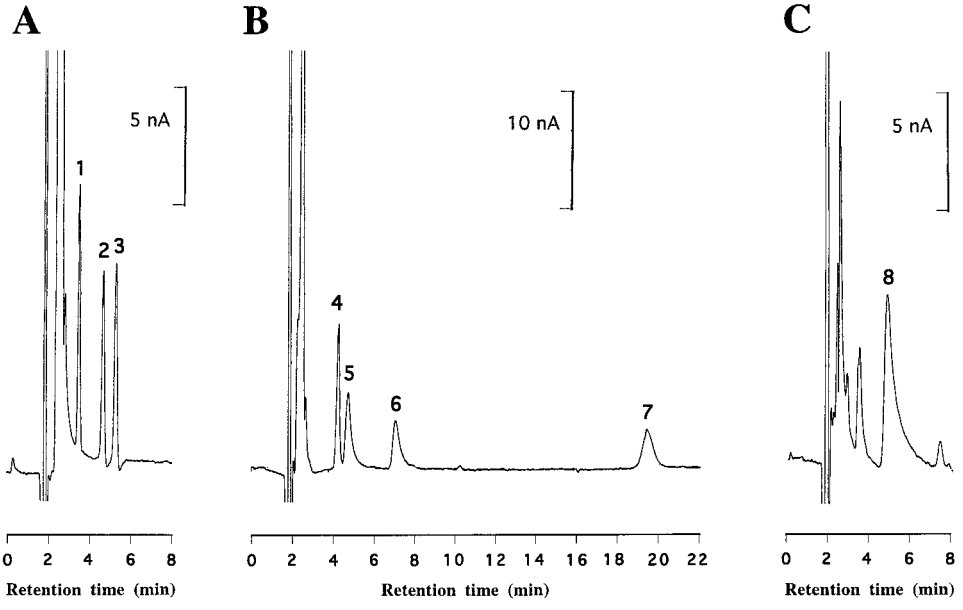

Fig. 1. Typical chromatograms of dyn-(1–8) and its seven hydrolysis products. The chromatograms were produced by applying 10 μl of the standard solution containing 10 pmol of each compound. The chromatographic mobile phase was 0.1 M phosphate buffer (pH 3.0) containing 2.5% acetonitrile for Y (1), YG (3) and YGG (2) assays (A); that containing 12% acetonitrile for YGGF (4), YGGFL (6), YGGFLR (5) and YGGFLRR (7) assays (B); and that containing 17% acetonitrile for dyn-(1–8) (8) assay (C), at the flow rate of 0.8 ml/min.

Calculations. The amount of each compound was determined by comparing the peak heights in the sample with that of the standard. Working standards were run at regular intervals to check the sensitivity of the detector. Y was always detected even when the membrane fraction sample was incubated in the absence of dyn-(1–8) for 60 min (Hiranuma and Oka, 1986; Hiranuma *et al.*, 1998). Therefore, the amount of Y in the absence of dyn-(1–8) was always subtracted from that in the presence of dyn-(1–8). In contrast to Y, dyn-(1–8) and its hydrolysis products other than Y were undetectable in any of the samples incubated in the absence of dyn-(1–8).

In *vitro* isolated preparation. Male ICR-Jcl mice weighing 40 to 50 g and male Hartley guinea pigs weighing 400 to 600 g were used for this study. The mouse vas deferens and the myenteric plexus-longitudinal muscle strip of guinea pig ileum were set up for electrical stimulation as described previously (Oka *et al.*, 1982). The % inhibition of the stimulated muscle twitch produced by an opioid was plotted against the log concentration of the opioid to estimate the IC_{50} (opioid concentration producing 50% inhibition of the twitch). When the effect of PIs on the IC_{50} value of an opioid peptide was studied, they were given 10 min before the administration of the opioid peptide. The K_e (equilibrium dissociation constant) values of opioid antagonists against the agonists were determined by the "single dose" method of Kosterlitz and Watt (1968). Because the IC_{50} value of an opioid in one preparation was sometimes significantly different from those in the others, the same preparation was used to carry out the experiment on the relative potency of opioids.

Results

The time course of the dyn-(1–8) hydrolysis and the generation of hydrolysis products during the incubation with membrane fractions. Dyn-(1–8) (5 nmol) was incubated at 37°C in 50 mM Tris-HCl buffer (pH 7.4) with a membrane fraction (0.5 mg of protein) prepared from either the myenteric plexus-longitudinal muscle strip of guinea pig ileum or the striatum of guinea pig brain. The amounts of dyn-(1–8) and its seven hydrolysis products: Y, YG, YGG, YGGF, YGGFL, YGGFLR and YGGFLRR in the reaction mixture were estimated at 0, 5, 15, 30, 45 and 60 min after starting the incubation. During the initial 5 min of incubation, dyn-(1–8) was hydrolyzed by approximately 44.4 and 41.4% in the ileal (fig. 2A) and striatal (fig. 2B) membrane fraction, respectively, and hydrolyzed almost completely at 60 min after starting the incubation in both membrane preparations (fig. 2A and B). The major hydrolysis products during the initial 5-min incubation were YGGFLR and Y in both preparations (fig. 2A and B), indicating that the dipeptidyl carboxypeptidase and aminopeptidase activities are mainly involved in the dyn-(1–8) hydrolysis.

The magnitude of the free Y generation in the ileal membrane fraction was higher than that in the striatal membrane fraction at any time of the estimation from 5 to 60 min after initiating the incubation (fig. 2A and B). In contrast to the Y formation, productions of both the YGGFLR and YGGF fragments in the ileal membrane fraction were lower than those in the striatal membrane fraction at any time between 5 to 45 min (fig. 2A and B). Additionally, the generation of the YG fragment in the ileal membrane fraction was lower than that in the striatal membrane fraction at any time between 30 to 60 min (fig. 2A and B).

Effects of the PIs on the dyn-(1–8) hydrolysis by the ileal membrane fraction. When the dyn-(1–8) (5 nmol) was incubated with the ileal membrane in the absence of PI for 60 min at 37°C, Y, YG, YGG and YGGF were detected in the sample, whereas YGGFL, YGGFLR, YGGFLRR and dyn-(1–8) were not (table 1). The percent production of Y, YG, YGG and YGGF was 69.4, 12.3, 13.8 and 2.5%, respectively (table 1). In contrast to the results without PI, the presence of 1 μM amastatin decreased the percent production of free Y to 1.0%; increased the percent productions of the YG, YGG and YGGF fragments to 44.9, 44.7 and 5.6%, respectively; and left 1.8% of the dyn-(1–8) intact (table 1). The presence of 1 μM captopril decreased the percent formation of both the YG and the YGGF fragments to 1.6 and 0.2%, respectively; increased both free Y and the YGG fragment to 73.9 and 16.5%, respectively; and left 6.1% of the dyn-(1–8) intact (table 1). In the presence of 1 μM phosphoramidon, the percent generation of the YGG fragment was decreased to 0.3%; percent generations of free Y, the YG and the YGGF fragments were increased to 74.7, 18.9 and 3.5%, respectively; and 0.4% of dyn-(1–8) remained intact (table 1). In the presence of both amastatin and captopril, the main hydrolysis product was the YGG fragment, and 36.2% of the dyn-(1–8)

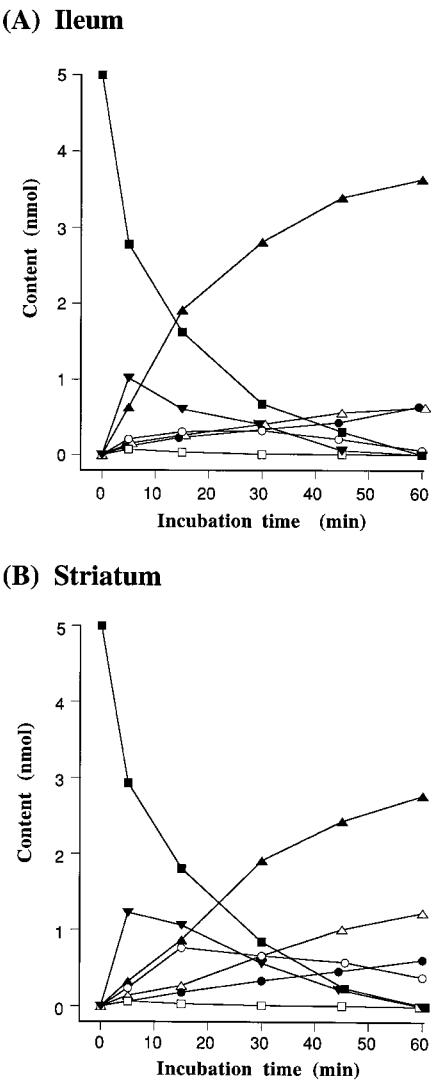

Fig. 2. The time course of the change in the amounts of dyn-(1–8) (■) and its six hydrolysis products: Y (▲), YG (△), YGG (●), YGGF (○), YGGFL (□) and YGGFLR (▼) in the reaction mixture during the incubation of dyn-(1–8) (5 nmol) with either the ileal (A) or striatal (B) membrane fraction (0.5 mg of protein each). Each point represents the mean of three determinations. Each S.E. was within 10% of the mean for both A and B.

TABLE 1
Effects of PIs on the hydrolysis of dyn-(1–8) incubated with an ileal membrane fraction

Inhibitors	% of Dyn-(1–8) and its Hydrolysis Products							
	Y	YG	YGG	YGGF	YGGFL	YGGFLR	YGGFLRR	Dyn-(1–8)
None	69.4 ± 0.6	12.3 ± 0.3	13.8 ± 0.2	2.5 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Ama	1.0 ± 0.1	44.9 ± 0.3	44.7 ± 0.3	5.6 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.8 ± 0.2
Cap	73.9 ± 0.3	1.6 ± 0.2	16.5 ± 0.5	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	6.1 ± 0.4
Pho	74.7 ± 0.5	18.9 ± 0.6	0.3 ± 0.1	3.5 ± 0.2	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.4 ± 0.1
Ama + Cap	1.1 ± 0.3	4.5 ± 0.2	56.2 ± 0.7	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	36.2 ± 0.3
Ama + Pho	1.2 ± 0.2	49.8 ± 0.3	0.2 ± 0.1	8.5 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	38.2 ± 0.4
Ama + Cap + Pho	1.0 ± 0.2	1.1 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	95.0 ± 0.3

The membrane fraction (0.5 mg of protein) in 50 mM Tris-HCl buffer (pH 7.4) was incubated with dyn-(1–8) (5 nmol) in either the absence or presence of the PIs at the final concentration of 1 μM each for 60 min at 37°C. Each value represents the mean ± S.E. of three experiments. Ama, amastatin; Cap, captopril; Pho, phosphoramidon.

remained intact (table 1). In the presence of both amastatin and phosphoramidon, the main hydrolysis product was the YG fragment, and 38.2% of the dyn-(1–8) remained intact (table 1). When amastatin, captopril and phosphoramidon were all present, 95.0% of the dyn-(1–8) remained intact (table 1).

Effects of the PIs on the hydrolysis of dyn-(1–8) by the striatal membrane fraction. The hydrolysis products of dyn-(1–8) formed during a 60-min incubation with the striatal membrane fraction were qualitatively similar to, but quantitatively different from, those yielded by the ileal membrane fraction in either the absence or the presence of the PIs (tables 1 and 2). The free Y generation in the absence of the PIs and the presence of either captopril or phosphoramidon after incubation with the striatal membrane fraction was lower than that observed with the ileal membrane fraction (tables 1 and 2). The formation of the YGG fragment in the absence of the PIs and the presence of amastatin after incubation of dyn-(1–8) with the striatal membrane fraction was lower than that with the ileal membrane fraction (tables 1 and 2). In contrast to the free Y and YGG fragment, the productions of both the YG and YGGF fragments in the absence of the PIs and the presence of phosphoramidon after incubation of dyn-(1–8) with the striatal membrane fraction were higher than that with the ileal membrane fraction (tables 1 and 2). However, the amount of dyn-(1–8) remaining intact 60 min after incubation with the striatal membrane fraction was higher than that with the ileal membrane fraction in the presence of either captopril alone or both amastatin and captopril (tables 1 and 2). In the presence of amastatin, captopril and phosphoramidon, however, 95% of the dyn-(1–8) remained intact after a 60-min incubation with either the ileal or the striatal membrane fraction (tables 1 and 2).

***K_e* values of *kappa* and *mu* antagonists against dyn-(1–8) in guinea pig ileum in either the absence or the presence of the three PIs.** The K_e values of nor-BNI, a selective *kappa* opioid receptor antagonist (Portoghese *et al.*, 1988), and CTOP, a selective *mu* antagonist (Gulya *et al.*, 1986; Pelton *et al.*, 1986), against morphine, a representative *mu* agonist, in guinea pig ileum were 24.8 and 18.2 nM, respectively, although those against ketocyclazocine, a representative *kappa* agonist, were 0.491 and 1,760 nM, respectively (table 3). The K_e values of nor-BNI and CTOP against dyn-(1–8) in guinea pig ileum in the absence of the PIs were 0.715 and 1,060 nM, respectively, while those in the presence of the three PIs were 0.226 and 824 nM, respectively (table 3). This indicates that dyn-(1–8) acts on *kappa* receptors in guinea pig ileum in both the absence and the presence of the three PIs. In contrast to dyn-(1–8), the K_e values of nor-BNI and CTOP against YGGFLR, a major hydrolysis product during the initial 5-min incubation (fig. 2A), in guinea pig ileum in the presence of the three PIs were 33.6 and 27.7 nM, respectively (table 3), indicating that YGGFLR acts on *mu* receptors in guinea pig ileum.

Potencies of dyn-(1–8) and its hydrolysis products in guinea pig ileum. We examined the potencies of ketocyclazocine and dyn-(1–17) relative to that of dyn-(1–8) and examined the potencies of the YGGFLR and YGGF fragments, the two hydrolysis products of dyn-(1–8), and morphine relative to that of DAMGO, a selective *mu* agonist (Handa *et al.*, 1981; Kosterlitz and Paterson, 1981) in guinea pig ileum pretreated with three PIs (amastatin, captopril and phosphoramidon) at the final concentration of 1 μM each. Pretreatment of the preparation with the three PIs did not change the respective IC_{50} value of ketocyclazocine, dyn-(1–17), morphine and DAMGO, but significantly decreased the respective IC_{50} value of dyn-(1–8), YGGFLR and YGGF in guinea pig ileum (data are not shown). In the presence of the three PIs, the potencies of dyn-(1–17) and ketocyclazocine were 271 and 78.2% that of dyn-(1–8), respectively (table 4), and the potencies of YGGFLR, YGGF which had been shown to act on *mu* receptors (Hiranuma *et al.*, 1997), and morphine

TABLE 2
Effects of PIs on the hydrolysis of dyn-(1–8) incubated with a striatal membrane fraction

Inhibitors	% of Dyn-(1–8) and its Hydrolysis Products							
	Y	YG	YGG	YGGF	YGGFL	YGGFLR	YGGFLRR	Dyn-(1–8)
None	51.3 ± 0.5	26.4 ± 0.6	11.2 ± 0.3	9.4 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Ama	0.4 ± 0.1	39.9 ± 0.4	38.7 ± 0.3	17.0 ± 0.5	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	2.2 ± 0.1
Cap	54.2 ± 0.3	2.3 ± 0.2	20.2 ± 0.4	0.3 ± 0.1	0.0 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	21.3 ± 0.4
Pho	55.8 ± 0.6	29.7 ± 0.3	0.1 ± 0.0	10.5 ± 0.2	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	2.6 ± 0.2
Ama + Cap	0.5 ± 0.2	2.8 ± 0.3	43.8 ± 0.4	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	50.7 ± 0.3
Ama + Pho	0.4 ± 0.2	40.2 ± 0.4	0.1 ± 0.0	18.6 ± 0.3	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	38.3 ± 0.4
Ama + Cap + Pho	0.6 ± 0.1	1.4 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	95.4 ± 0.5

The membrane fraction (0.5 mg of protein) in 50 mM Tris-HCl buffer (pH 7.4) was incubated with dyn-(1–8) (5 nmol) in either the absence or presence of the PIs at the final concentration of 1 μM each for 60 min at 37°C. Each value represents the mean ± S.E. of three experiments. Ama, amastatin; Cap, captopril; Pho, phosphoramidon.

TABLE 3
The Ke values of nor-BNI and CTOP against dyn-(1–8), YGGFLR, and representative *mu* and *kappa* agonists in guinea pig ileum in either the absence (−) or presence (+) of three PIs

Opioid agonists	PIs	Ke (nM)			
		n	Nor-BNI	*n*	CTOP
Dyn-(1–8)	+	6	0.226 ± 0.016	10	824 ± 180
Dyn-(1–8)	−	6	0.715 ± 0.23	4	1060 ± 230
YGGFLR	+	7	33.6 ± 2.7	5	27.7 ± 2.6
Morphine	−	4	24.8 ± 2.4	8	18.2 ± 1.8
Ketocyclazocine	−	4	0.491 ± 0.060	4	1760 ± 320

The mixture of three PIs, amastatin, captopril and phosphoramidon, at the final concentration of 1 μM each was given 10 min before the opioid peptide administration. The opioid antagonist was given 5 min before the agonist injection. Each value represents the mean ± S.E. of *n* experiments.

TABLE 4
The potencies of dyn-(1–8) and its hydrolysis products relative to those of representative agonists at *kappa* and *mu* opioid receptors in guinea-pig ileum pretreated with three PIs

Opioid Agonists	*n*	IC50 (nM)	Relative Potency
Dyn-(1–8)	4	2.54 ± 0.11	1
Dyn-(1–17)	4	0.948 ± 0.085	2.71 ± 0.14
Ketocyclazocine	4	3.26 ± 0.18	0.782 ± 0.035
DAMGO	8	12.8 ± 1.8	1
YGGFLR	8	48.4 ± 7.5	0.273 ± 0.018
YGGF	8	2110 ± 400	0.00686 ± 0.00097
DAMGO	4	7.81 ± 1.3	1
Morphine	4	95.3 ± 20	0.0839 ± 0.0044

The mixture of three PIs, amastatin, captopril and phosphoramidon, at the final concentration of 1 μM each was given 10 min before the opioid administration. Each value represents the mean ± S.E. of *n* experiments.

were 27.3, 0.686 and 8.39% that of DAMGO, respectively (table 4).

Effects of PIs on the K_e values of *kappa* and *delta* antagonists against dyn-(1–8) in mouse vas deferens. The K_e values of nor-BNI and ICI-174864, a selective *delta* opioid receptor antagonist (Cotton *et al.*, 1984), against dyn-(1–8) in mouse vas deferens were, respectively, 2.08 and 35.9 nM in the absence of any of the PIs; 0.0906 and 18,800 nM in the presence of captopril; 1.92 and 23.2 nM in the presence of both amastatin and phosphoramidon; and 0.0556 and 13,500 nM in the presence of the three PIs (table 5). Additionally, the K_e values of nor-BNI and ICI-174864 against the YGGFLR fragment in mouse vas deferens were 3.85 and 11.4 nM, respectively, in the presence of the three PIs, indicating that the YGGFLR fragment acts on *delta* receptors in mouse vas deferens. The results indicate that in mouse vas deferens, dyn-(1–8) acts on *kappa* receptors in the presence of captopril, but in the absence of captopril, a large part of the dyn-(1–8) is hydrolyzed to the YGGFLR fragment, which then acts on *delta* receptors.

Discussion

The hydrolysis of dyn-(1–8) has been examined by incubating it with the membrane fraction prepared from either the myenteric plexus-longitudinal muscle strip of guinea pig ileum or guinea pig striatum in either the absence or presence of the PIs. Our results show that dyn-(1–8) is completely hydrolyzed during the 60-min incubation in the absence of the three PIs, but in their presence, its hydrolysis is almost completely prevented. Until now, the potency of dyn-(1–8) has not been accurately estimated due to its susceptibility to degradation. In our study, by using the three PIs to eliminate hydrolytic inactivation of dyn-(1–8), we were able to determine its potency and compare it to those of representative opioids.

The fact that the major hydrolysis product of dyn-(1–8) is the YGGFLR fragment during the first 5 min of incubation in both the ileal and striatal membrane fractions indicates that the dipeptidyl carboxypeptidase activity is mainly involved in the hydrolysis of dyn-(1–8). Additionally, the observation that the amount of the YGGFLR fragment decreases while that of the YGGF fragment concomitantly increases from 5 to 15 min after initiation of the incubation indicates that the YGGFLR and the YGGF fragments are sequentially produced in this order by the dipeptidyl carboxypeptidase.

The dipeptidyl carboxypeptidase activity in both membrane fractions can be identified as CsD by the fact that YGGF fragment generation during the 60-min incubation was almost completely prevented by the presence of captopril, a specific inhibitor of dipeptidyl carboxypeptidase I (Rubin *et al.*, 1978). Similarly, the enzyme activity producing the free Y and the YGG fragment can be identified as AsA and PsE, respectively, because the productions of the free Y and YGG fragment were almost completely prevented by amastatin, a potent inhibitor of aminopeptidase (Matsas *et al.*, 1985), and phosphoramidon, a specific inhibitor of endopeptidase-24.11 (Matsas *et al.*, 1983), respectively.

When dyn-(1–8) is incubated with the ileal or striatal

TABLE 5
Effects of peptidase inhibitors on the Ke values of nor-BNI and ICI-174864 against dyn-(1–8) in mouse vas deferens

Peptidase inhibitors	Ke (nM)			
	n	Nor-BNI	*n*	ICI-174864
None	4	2.08 ± 0.15	4	35.9 ± 2.5
Cap	4	0.0906 ± 0.015	4	18800 ± 3200
Ama + Pho	8	1.92 ± 0.31	8	23.2 ± 3.0
Ama + Cap + Pho	6	0.0556 ± 0.0034	6	13500 ± 3100

Captopril (Cap) or the mixture of two or three of the following PIs: amastatin (Ama), phosphoramidon (Pho) and Cap, at the final concentration of 1 μM each, was given 10 min before the dyn-(1–8) administration. Each value represents the mean ± S.E. of *n* experiments.

membrane fraction for 60 min in the presence of captopril, 74 or 54%, respectively, of dyn-(1–8) is hydrolyzed to the free Y. This indicates that when CsD is inhibited, dyn-(1–8) is hydrolyzed mainly by AsA. Additionally, when dyn-(1–8) is incubated with the ileal or striatal membrane fraction for 60 min in the presence of both captopril and amastatin, 56 or 44%, respectively, of dyn-(1–8) is hydrolyzed to the YGG fragment. This indicates that when both CsD and AsA are inhibited, PsE is then able to hydrolyze a significant amount of dyn-(1–8). The involvement of PsE in the hydrolysis of dyn-(1–8) is also supported by the observation that when enkephalin octapeptide is incubated with the ileal or striatal membrane fraction for 60 min in the presence of the three PIs, amastatin, captopril and phosphoramidon, approximately 95 or 95%, respectively, of the dyn-(1–8) remains intact.

Because the hydrolysis products of dyn-(1–8) by either AsA or PsE such as free Y and the YGG, [des-Y]-dyn-(1–8) and [des-YGG]-dyn-(1–8) fragments are suggested to have very low, if any, agonist activity against opioid receptors (Morley, 1980), the potency of dyn-(1–8) is expected to be decreased by its hydrolysis by these two peptidases. In fact, the potency of dyn-(1–8) in guinea pig ileum has been shown to be significantly increased by either amastatin or phosphoramidon in the previous investigation (Numata *et al.*, 1988). Additionally, the pharmacological evidence obtained in the previous study (Numata *et al.*, 1988) showing that the magnitude of the enhancement of the dyn-(1–8) potency by amastatin is significantly higher than that by phosphoramidon is consistent with the present biochemical data indicating that AsA is involved in the dyn-(1–8) hydrolysis to a significantly higher degree than PsE.

In contrast to the hydrolysis products of dyn-(1–8) by AsA and PsE, the YGGFLR fragment, the initial hydrolysis product of dyn-(1–8) by CsD, still has the high agonist activity on opioid receptors. The K_e values of highly selective *mu*, *kappa* and *delta* antagonists against YGGFLR indicate that YGGFLR acts on *mu* receptors in guinea pig ileum and *delta* receptors in mouse vas deferens. The K_e values of ICI-174864 and nor-BNI against dyn-(1–8) indicate that dyn-(1–8) acts on *delta* receptors in mouse vas deferens in the absence of captopril although it acts on *kappa* receptors in the presence of captopril. These results suggest that in mouse vas deferens, a large part of exogenously added dyn-(1–8) must be hydrolyzed to the YGGFLR fragment, which then interacts with *delta* receptors, in the absence of captopril. In contrast to mouse vas deferens, the K_e value of nor-BNI against dyn-(1–8) indicates that dyn-(1–8) acts on *kappa* receptors in guinea pig ileum in both the presence and the absence of captopril. This indicates that in guinea pig ileum, the ratio of dyn-(1–8), which acts on *kappa* receptors, to YGGFLR, which acts on *mu* receptors, is high at the vicinity of opioid receptors due to the low activity of CsD. The present results obtained by employing the two isolated preparations strongly suggest that endogenous dyn-(1–8) released from the nerve cell acts through dyn-(1–8) itself or its main hydrolysis product, YGGFLR, on either *kappa*, *mu* or *delta* opioid receptors, depending on both the relative activity of the three enzymes, AsA, CsD and PsE, at the vicinity of opioid receptors and the relative density of the three opioid-receptor types, *kappa*, *mu* and *delta* receptors, at the synaptic membrane.

The electrically evoked contractions of guinea pig ileum were shown to be inhibited by *mu* and *kappa* agonists but not by *delta* agonists (Lord *et al.*, 1977). In our study, we showed that dyn-(1–8) acts on the *kappa* receptors, being consistent with the previous findings (Corbett *et al.*, 1982; Oka and Negishi, 1982), although its hydrolysis products, the YGGFLR and YGGF fragments, act on the *mu* receptors in guinea pig ileum pretreated with the three PIs. Because the potency of dyn-(1–8) has yet to be estimated under conditions where its degradation is completely prevented, we determined its potency and compared it with those of representative *kappa* agonists in guinea pig ileum pretreated with three PIs, a condition that totally prevents hydrolysis. The present results show that the rank order of potencies is as follows: dyn-(1–17) > dyn-(1–8) > ketocyclazocine.

Additionally, we determined the potencies of the hydrolysis products of dyn-(1–8) and compared them with those of representative *mu* agonists in guinea pig ileum pretreated with the three PIs. Our results taken together with previous ones (Hiranuma *et al.*, 1997) show that the rank order of potencies is as follows: YGGFMR > DAMGO > met-enk-RGL > YGGFLR > morphine ≫ YGGF. Moreover, our preliminary experiments showed that the potency of YGGFLR was 45% that of [D-Pen^{2,5}]-enkephalin, a representative potent *delta* agonist (Mosberg *et al.*, 1983), in mouse vas deferens in which YGGFLR was shown to act on *delta* receptors, in our study. These results show that the YGGFLR fragment, the initial hydrolysis product of dyn-(1–8), still has the ability to act as a potent agonist on either *mu* or *delta* opioid receptors.

The data obtained in the previous studies in which the effects of three PIs, amastatin, captopril and phosphoramidon, on the hydrolysis of met-enk (Hiranuma and Oka, 1986) and met-enk-RGL (Hiranuma *et al.*, 1997) were investigated, together with those obtained in the present investigation show that three endogenous opioid peptides, met-enk, met-enk-RGL and dyn-(1–8), are almost exclusively hydrolyzed by three distinct enzymes, AsA, CsD and PsE, in both ileal and striatal membrane fractions. Additionally, our recent experiment (Hiranuma *et al.*, 1998) showed that the hydrolysis of [Leu^5]-enkephalin and [Met^5]-enkephalin-Arg^6-Phe^7 in the membrane fractions is also almost completely prevented by the mixture of these three PIs. Because all three enzymes have been indicated to be very closely located to opioid receptors in previous studies (Aoki *et al.*, 1984, 1986; Cui *et al.*, 1986; Numata *et al.*, 1988), they must play critical roles in the physiological inactivation of several endogenous opioid peptides. Interestingly, all three enzymes are also indicated to have important functions in the inactivation of opioid peptides after their systemic administration by the fact that the s.c. administration of met-enk is shown to produce two naloxone-reversible effects: inhibition of the tail-flick response and loss of the righting reflex in 10-day-old rats pretreated with all three PIs, but not in those pretreated with any combination of two PIs (Oka *et al.*, 1992).

Acknowledgments

The authors thank Dr. S. M. Jung, Institute of Life Science, Kurume University, Kurume, Japan, for helpful comments and thank Mrs. Kazuko Aoki for her expert technical assistance. The generous gifts of ketocyclazocine and captopril described in "Materials and Methods" are gratefully acknowledged.

References

Aoki K, Kajiwara M and Oka T (1984) The role of bestatin-sensitive aminopeptidase, angiotensin converting enzyme and thiorphan-sensitive "enkephalinase" in the potency of enkephalins in the guinea-pig ileum. *Jpn J Pharmacol* **36:**59–65.

Aoki K, Kajiwara M and Oka T (1986) The inactivation of [Met^5]-enkephalin by bestatin-sensitive aminopeptidase, captopril-sensitive peptidyl dipeptidase A and thiorphan-sensitive endopeptidase-24.11 in mouse vas deferens. *Jpn J Pharmacol* **40:**297–302.

Corbett AD, Paterson SJ, McKnight AT, Magnan J and Kosterlitz HW (1982) Dynorphin$_{1-8}$ and Dynorphin$_{1-9}$ are ligands for the κ-subtype of opiate receptor. *Nature* **299:**79–81.

Cotton R, Giles MG, Miller L, Shaw JS and Timms D (1984) ICI 174864: A highly selective antagonist for the opioid δ-receptor. *Eur J Pharmacol* **97:**331–332.

Cui S, Kajiwara M, Ishii K, Aoki K, Sakamoto J, Matsumiya T and Oka T (1986) The enhancing effects of amastatin, phosphoramidon and captopril on the potency of [Met^5]-enkephalin in rat vas deferens. *Jpn J Pharmacol* **42:**43–49.

Glowinski J and Iversen LL (1966) Regional studies of catecholamines in the rat brain-1: the disposition of [^{3}H]norepinephrine, [^{3}H]dopamine and [^{3}H]dopa in various regions of the brain. *J Neurochem* **13:**655–669.

Gulya K, Pelton JT, Hruby VJ and Yamamura HI (1986) Cyclic somatostatin octapeptide analogues with high affinity and selectivity toward mu opioid receptors. *Life Sci* **38:**2221–2229.

Handa BK, Lane AC, Lord JAH, Morgan BA, Rance MJ and Smith CFC (1981) Analogues of β-LPH$_{61-64}$ possessing selective agonist activity at μ-opiate receptors. *Eur J Pharmacol* **70:**531–540.

Hiranuma T, Iwao K, Kitamura K, Matsumiya T and Oka T (1997) Almost complete protection from [Met^5]-enkephalin-Arg^6-Gly^7-Leu^8 (Met-enk-RGL) hydrolysis in membrane preparations by the combination of amastatin, captopril and phosphoramidon. *J Pharmacol Exp Ther* **281:**769–774.

Hiranuma T, Kitamura K, Taniguchi T, Kobayashi T, Tamaki R, Kanai M, Akahori K, Iwao K and Oka T (1998) Effects of three peptidase inhibitors, amastatin, captopril and phosphoramidon, on the hydrolysis of [Met^5]-enkephalin-Arg^6-Phe^7 and other opioid peptides. *Naunyn-Schmiedeberg Arch Pharmacol*, **357:**276–282.

Hiranuma T and Oka T (1986) Effects of peptidase inhibitors on the [Met^5]-enkephalin hydrolysis in ileal and striatal preparations of guinea-pig: almost complete protection of degradation by the combination of amastatin, captopril and thiorphan. *Jpn J Pharmacol* **41:**437–446.

Kakidani H, Furutani Y, Takahashi H, Noda M, Morimoto Y, Hirose T, Asai M, Inayama S, Nakanishi S and Numa S (1982) Cloning and sequence analysis of cDNA for porcine β-neo-endorphin/dynorphin precursor. *Nature* **298:**245–249.

Kosterlitz HW and Paterson SJ (1981) Tyr-D-Ala-Gly-MePhe-NH(CH$_2$)$_2$OH is a selective ligand for the μ-opiate binding site. *Br J Pharmacol* **73:**299P.

Kosterlitz HW and Watt AJ (1968) Kinetic parameters of narcotic agonists and antagonists, with particular reference to N-allylnoroxymorphone (naloxone). *Br J Pharmacol* **33:**266–276.

Lord JAH, Waterfield AA, Hughes J and Kosterlitz HW (1977) Endogenous opioid peptides: multiple agonists and receptors. *Nature* **267:**495–499.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:**265–275.

Matsas R, Fulcher IS, Kenny AJ and Turner AJ (1983) Substance P and [Leu]enkephalin are hydrolyzed by an enzyme in pig caudate synaptic membranes that is identical with the endopeptidase of kidney microvilli. *Proc Natl Acad Sci USA* **80:**3111–3115.

Matsas R, Stephenson SL, Hryszko J, Kenny AJ and Turner AJ (1985) The metabolism of neuropeptides. *Biochem J* **231:**445–449.

McKnight AT, Corbett AD and Kosterlitz HW (1983) Increase in potencies of opioid peptides after peptidase inhibition. *Eur J Pharmacol* **86:**393–402.

Morley JS (1980) Structure-activity relationships of enkephalin-like peptides. *Annu Rev Pharmacol Toxicol* **20:**81–110.

Mosberg HI, Hurst R, Hruby VJ, Gee K, Yamamura HI, Galligan JJ and Burks TF (1983) Bis-penicillamine enkephalins possess highly improved specificity toward δ opioid receptors. *Proc Natl Acad Sci USA* **80:**5871–5874.

Numata H, Hiranuma T and Oka T (1988) Inactivation of dynorphin-(1–8) in isolated preparations by three peptidases. *Jpn J Pharmacol* **47:**417–423.

Oka T, Liu X-F, Kajita T, Ohgiya N, Ghoda K, Taniguchi T, Arai Y and Matsumiya T (1992) Effects of the subcutaneous administration of enkephalins on tail-flick response and righting reflex of developing rats. *Dev Brain Res* **69:**271–276.

Oka T and Negishi K (1982) Evidence that endogenous 6-(Arg or Lys)-opioid peptides can interact with κ-receptors as agonists. *Life Sci* **31:**1707–1710.

Oka T, Negishi K, Suda M, Sawa A, Fujino M and Wakimasu M (1982) Evidence that dynorphin-(1–13) acts as an agonist on opioid κ-receptors. *Eur J Pharmacol* **77:**137–141.

Pelton JT, Kazmierski W, Gulya K, Yamamura HI and Hruby VJ (1986) Design and synthesis of conformationally constrained somatostatin analogues with high potency and specificity for μ opioid receptors. *J Med Chem* **29:**2370–2375.

Portoghese PS, Nagase H, Lipkowski AW, Larson DL and Takemori AE (1988) Binaltorphimine-related bivalent ligands and their κ opioid receptor antagonist selectivity. *J Med Chem* **31:**836–841.

Rubin B, Laffan RJ, Kotler DG, O'Keefe EH, Demaio DA and Goldberg ME (1978) SQ 14,225 (D-3-mercapto-2-methyl proparoyl-L-proline), a novel orally active inhibitor of angiotensin I converting enzymes. *J Pharmacol Exp Ther* **204:**271–280.

Send reprint requests to: Dr. Tetsuo Oka, Department of Pharmacology, School of Medicine, Tokai University, Isehara 259-11, Japan.