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

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ABSTRACT

The amounts of dynorphin-(1–8) [dyn-(1–8)] and its seven hydrolysis products, Y, YG, YGG, YGGF, 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 K(e) (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: [Met5]-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-Arg6-Gly7-Leu8 (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 opioid peptides include dipeptidyl carboxypeptidase IIA, aminopeptidase N and aminopeptidase M, which have been shown to be located on the same membrane fraction, in addition to dipeptidyl carboxypeptidase I (AsA), aminopeptidase N and aminopeptidase M (PsE) and aminopeptidase M (CsD) (Chattopadhyay et al., 1989). This suggests that the hydrolysis of endogenous opioid peptides is mediated by a combination of these enzymes.

ABBREVIATIONS: dyn-(1–8) and -(1–17), dynorphin-(1–8) and -(1–17); AsA, amastatin-sensitive aminopeptidase(s); PsE, phosphoramidon-sensitive endopeptidase-24.11 (“enkephalase”, EC 3.4.24.11); CsD, captopril-sensitive dipeptidyl carboxypeptidase I (angiotensin 1 converting enzyme, kininase II, EC 3.4.15.1); met-enk, [Met5]-enkephalin; met-enk-RGL, [Met5]-enkephalin-Arg6-Gly7-Leu8; PI, peptidase inhibitor; DAMGO, [a-Ala2, N-Me-Phe4,Gly-ol]-enkephalin; DPDPE, [a-Pen2]-enkephalin; CTP, ß-Phe-Cys-Tyr-ß-Trp-Om-Thr-Pen-Thr-NH2; nor-BNI, nor-binaltorphimine HCl tetrahydrate; IC174,864, N,N-diallyl-Tyr-Alb-Alb-Phe-Leu-OH (Alb = a-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 agonists. However, they have not been thoroughly 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, DPDPDE and CTP (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 (Leu5)-enkephalin, dynorphin-(1–17) [dyn-(1–17)], amastatin and phosphoramidon (Peptide Institute, Inc., Minoh, Japan); YG, 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) (ICl-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 siliconeizing 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 μBondapak sphere 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, YGGFLR and YGGFLRR (fig. 1B); and 17% acetonitrile 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, YGGFL, YGGFLR and YGGFLRR and dyn-(1–8) exhibited a retention time of approximately 3.58, 5.38, 4.77, 4.23, 19.6, 7.10 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 was 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 IC50 (opioid concentration producing 50% inhibition of the twitch). When the effect of PIs on the IC50 value of an opioid peptide was studied, they were given 10 min before the administration of the opioid peptide. The Kd (equilibrium dissociation constant) values of opioid antagonists against the agonists were determined by the “single dose” method of Kosterlitz and Watt (1968). Because the IC50 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 YGGF 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)
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 lower than that 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) formed during a 60-min incubation with the striatal membrane fraction were quantitatively similar to, but lower than that 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 ileal Y and YGG fragment, the production 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 (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 the ileal or the striatal membrane fraction (tables 1 and 2).

**Kₐ 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 Kₐ 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 Kₐ 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 Kₐ 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 IC₅₀ value of ketocyclazocine, dyn-(1–17), morphine and DAMGO, but significantly decreased the respective IC₅₀ 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

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**TABLE 1**

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

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Y</th>
<th>YG</th>
<th>YGG</th>
<th>YGGF</th>
<th>YGGFL</th>
<th>YGGFLR</th>
<th>Dyn-(1–8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>51.3 ± 0.5</td>
<td>26.4 ± 0.6</td>
<td>11.2 ± 0.3</td>
<td>9.4 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Ama</td>
<td>64.9 ± 0.6</td>
<td>12.3 ± 0.3</td>
<td>13.8 ± 0.2</td>
<td>2.5 ± 0.4</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Pho</td>
<td>74.7 ± 0.5</td>
<td>18.9 ± 0.6</td>
<td>0.2 ± 0.1</td>
<td>3.5 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Ama + Pho</td>
<td>1.1 ± 0.3</td>
<td>4.5 ± 0.2</td>
<td>56.2 ± 0.7</td>
<td>0.2 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Ama + Pho</td>
<td>1.2 ± 0.2</td>
<td>49.8 ± 0.3</td>
<td>0.2 ± 0.1</td>
<td>8.5 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

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.
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 0.0565 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 3.08 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.

### TABLE 3

The $K_e$ 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.

<table>
<thead>
<tr>
<th>Opioid agonists</th>
<th>PIs</th>
<th>$n$</th>
<th>Nor-BNI</th>
<th>CTOP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dyn-(1–8)</td>
<td>+</td>
<td>6</td>
<td>0.226 ± 0.016</td>
<td>10</td>
</tr>
<tr>
<td>Dyn-(1–8)</td>
<td>-</td>
<td>6</td>
<td>0.715 ± 0.23</td>
<td>4</td>
</tr>
<tr>
<td>YGGFLR</td>
<td>+</td>
<td>7</td>
<td>33.6 ± 2.7</td>
<td>5</td>
</tr>
<tr>
<td>Morphine</td>
<td>-</td>
<td>4</td>
<td>24.8 ± 2.4</td>
<td>8</td>
</tr>
<tr>
<td>Ketocyclazocine</td>
<td>-</td>
<td>4</td>
<td>0.491 ± 0.060</td>
<td>4</td>
</tr>
</tbody>
</table>

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.

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

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.

### TABLE 5

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

<table>
<thead>
<tr>
<th>Peptidase inhibitors</th>
<th>$n$</th>
<th>Nor-BNI</th>
<th>ICI-174864</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4</td>
<td>2.08 ± 0.15</td>
<td>35.9 ± 2.5</td>
</tr>
<tr>
<td>Cap</td>
<td>4</td>
<td>0.0906 ± 0.015</td>
<td>18800 ± 3200</td>
</tr>
<tr>
<td>Ama + Pho</td>
<td>8</td>
<td>1.92 ± 0.31</td>
<td>23.2 ± 3.0</td>
</tr>
<tr>
<td>Ama + Cap + Pho</td>
<td>6</td>
<td>0.0556 ± 0.0034</td>
<td>13500 ± 3100</td>
</tr>
</tbody>
</table>

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.

### 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 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 YGGF fragment can be identified as AsA and PsE, respectively, because the productions of the free Y and YGGF 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...
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 hy-
drolyzed 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, approxi-
mately 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 signifi-
cantly increased by either amastatin or phosphoramidon in
the previous investigation (Numata et al., 1988). Addition-
ally, 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 consis-
tent 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 prod-
uct of dyn-(1–8) by CsD, still has the high agonist activity on
opioid receptors. The Ke values of highly selective mu, kappa
and delta antagonists against YGGFLR indicate that YGG-
FLR acts on mu receptors in guinea pig ileum and delta
receptors in mouse vas deferens. The Ke 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 defer-
ens, 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 Ke 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 recep-
tors 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 prod-
uct, 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 YGG-
FLR 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 re-
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 rep-
resentative 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 > YGG-
FLR > morphine > YGGF. Moreover, our preliminary ex-
periments showed that the potency of YGGFLR was 45% that
of [D-Pen2,5]-enkephalin, a representative potent delta ago-
nist (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 phosphorami-
don, 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 hydro-
lysis of [Leu5]-enkephalin and [Met5]-enkephalin-Arg6-Pho7
in the membrane fractions is also almost completely pre-
vented 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 pro-
duce two naloxone-reversible effects: inhibition of the tail-
lick 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).

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