The Relative Potency of Enkephalins and β-Endorphin in Guinea-Pig Ileum, Mouse Vas Deferens and Rat Vas Deferens after the Administration of Peptidase Inhibitors

Yoshiki KUNO, Kazuko AOKI, Midori KAJIWARA, Kaori ISHII and Tetsuo OKA*

Department of Pharmacology, School of Medicine, Tokai University, Isehara 259-11, Japan
Accepted March 17, 1986

Abstract—Previous studies have shown that three distinct enzymes, amastatin-sensitive aminopeptidase, captopril-sensitive peptidyl dipeptidase A, and phosphoramidon-sensitive endopeptidase-24.11, played a critical role in the inactivation of enkephalins in isolated preparations. In the present study, therefore, the rank order of the potency of three endogenous opioid peptides, [Met\(^5\)]-enkephalin, [Leu\(^5\)]-enkephalin, and β-endorphin, in three isolated preparations, guinea-pig ileum, mouse vas deferens, and rat vas deferens, was estimated in the presence of the mixture of three peptidase inhibitors, amastatin, captopril, and phosphoramidon. [Met\(^5\)]-Enkephalin was approximately three-fold more potent than [Leu\(^5\)]-enkephalin and four-fold more potent than β-endorphin in guinea-pig ileum in which three opioid peptides were indicated to act on mu-receptors. Additionally, [Met\(^5\)]-enkephalin was slightly but significantly more potent than [Leu\(^5\)]-enkephalin and approximately twenty-fold more potent than β-endorphin at delta-receptor sites in mouse vas deferens. Moreover, [Met\(^5\)]-enkephalin was approximately three-fold more potent than [Leu\(^5\)]-enkephalin, but sixty-fold less potent than β-endorphin in rat vas deferens in which the opioid-receptor type interacting with enkephalins could not be determined. In conclusion, the well-known rank order of the potency of three endogenous opioid peptides was shown to be altered in both guinea-pig ileum and mouse vas deferens but not in rat vas deferens by the pretreatment of the preparations with the mixture of three peptidase inhibitors.

It is well-known that [Met\(^5\)]-enkephalin is more potent than [Leu\(^5\)]-enkephalin in guinea-pig ileum, while the latter is more potent than the former in mouse vas deferens (1, 2). Additionally, it is reported that β-endorphin has either approximately the same potency as, or slightly higher potency than, [Met\(^5\)]-enkephalin in guinea-pig ileum, while the latter is more potent than the former in mouse vas deferens (3, 4). Moreover, β-endorphin is shown to be about a thousand-fold more potent than [Met\(^5\)]-enkephalin in rat vas deferens (5, 6). The above-mentioned fact that the rank order of the potency of opioid peptides in one isolated preparation is different from that in the other preparation provides important evidence that the receptor populations in one preparation are not identical to those in the other preparation. These studies on the potency of opioid peptides, however, have been carried out in the absence of peptidase inhibitors.

The combination of peptidase inhibitors has been reported to increase the potency of opioid peptides in isolated preparations (7). Additionally, three distinct enzymes, both bestatin- and amastatin-sensitive aminopeptidase, captopril-sensitive peptidyl dipeptidase A, and phosphoramidon-sensitive endopeptidase-24.11, played a critical role in the inactivation of enkephalins in isolated preparations. In the present study, therefore, the rank order of the potency of three endogenous opioid peptides was estimated in the presence of the mixture of three peptidase inhibitors, amastatin, captopril, and phosphoramidon.
peptidase A, and both thiorphan- and phosphoramidon-sensitive endopeptidase-24.11, have been shown to inactivate the enkephalins in three isolated preparations, guinea-pig ileum (8), mouse vas deferens (9), and rat vas deferens (manuscript in preparation). Since the peptidases involved in the inactivation of opioid peptides may differ in their substrate preference, the rank order of the potency of opioid peptides obtained in the absence of peptidase inhibitors may be different from that in the presence of peptidase inhibitors.

In the present study, therefore, the rank order of the potency of three endogenous opioid peptides, [Met\(^5\)]-enkephalin, [Leu\(^5\)]-enkephalin, and \(\beta\)-endorphin, in three isolated preparations, guinea-pig ileum, mouse vas deferens, and rat vas deferens, was estimated in the presence of three peptidase inhibitors, amastatin, captopril and phosphoramidon.

### Materials and Methods

**Chemicals:** Gifts of compounds which were gratefully received were captopril and naloxone-HCl from Sankyo Company (Tokyo); amastatin and phosphoramidon from Dr. T. Aoyagi, Institute of Microbial Chemistry (Tokyo); Mr 2266 [(−)-2-(3-furylmethyl)-5,9-diethyl-2'-hydroxy-6,7-benzomorphan] from Nippon C.H. Boehringer Sohn Co., Ltd. (Osaka); ICI 154129 (N,N-diallyl-Tyr-Gly-Gly-o-(CH\(_2\)S)Phe-Leu) [where O-(CH\(_2\)S) signifies replacement of the amide CO-NH bond by CH\(_2\)S] from Dr. J.W. Holaday, Walter Reed Army Inst. Res. (Washington, D.C., U.S.A.); and \(\beta\)-endorphin from Dr. M. Fujino, Central Research Division, Takeda Chemical Ind., Ltd. (Osaka). [Met\(^5\)]-Enkephalin and [Leu\(^5\)]-enkephalin were purchased from the Peptide Institute, Inc. (Minoh).

**In vitro isolated preparations:** Male ICR-JCL mice weighing 30–50 g, male Wistar rats weighing 200–400 g, and male Hartley guinea-pigs weighing 300–500 g were used for this study. The vasa deferentia from mice or rats, and the myenteric plexus-longitudinal muscle strip of guinea-pig ileum were prepared and set up for electrical stimulation as described previously (10). The % inhibition of the stimulated muscle twitch produced by an opioid was plotted against the log concentration of the opioid to estimate the IC50 (concentration of the opioid peptide to produce 50% inhibition of the twitch). When the effect of peptidase inhibitors on the IC50 value of an opioid peptide was studied, they were given at least ten minutes before the administration of the opioid peptide. The K\(_e\) (equilibrium dissociation constant) value of naloxone, Mr 2266 or ICI 154129 against opioid peptides was determined by the 'single dose' method of Kosterlitz and Watt (11). Since the potency of an opioid in one preparation was sometimes significantly different from the others, the experiment on the relative potency of opioid peptides was carried out by employing the same preparation.

### Results

**Inhibitory potency of opioid peptides in isolated preparations in the absence of peptidase inhibitors:** Endogenous opioid peptides such as [Met\(^5\)]-enkephalin, [Leu\(^5\)]-enkephalin and \(\beta\)-endorphin significantly inhibited the electrically-evoked contractions of either guinea-pig ileum, mouse vas deferens or rat vas deferens in the absence of peptidase inhibitors. In guinea-pig ileum, \(\beta\)-endorphin was approximately two times more potent than [Met\(^5\)]-enkephalin which was about three-fold more potent than [Leu\(^5\)]-enkephalin (Table 1). Additionally, in mouse vas deferens, [Leu\(^5\)]-enkephalin was approximately two times more potent than [Met\(^5\)]-enkephalin which was about two times more potent than \(\beta\)-endorphin (Table 1). Moreover, \(\beta\)-endorphin was approximately fifteen hundred-fold more potent than [Met\(^5\)]-enkephalin which was about two times more potent than [Leu\(^5\)]-enkephalin in rat vas deferens (Table 1).

On the other hand, the inhibitory potency of both [Met\(^5\)]-enkephalin and [Leu\(^5\)]-enkephalin was the highest in mouse vas deferens, the lowest in rat vas deferens, and intermediate in guinea-pig ileum (Tables 1, 2 and 3). In contrast to enkephalins, the potency of \(\beta\)-endorphin was the highest in rat vas deferens, the lowest in guinea-pig ileum, and intermediate in mouse vas deferens.
Table 1. The potency of [Leu⁵]-enkephalin (LE) or β-endorphin (β-End) relative to that of [Met⁵]-enkephalin (ME) in isolated preparations in the absence of peptidase inhibitors

| Preparations   | N | IC50 (nM)     | Relative potency<sup>h</sup> |
|---------------|---|---------------|-----------------------------|
|               |   | ME | LE | β-End |                        |
| Guinea-pig ileum | 4 | 564 ± 180 | 2070 ± 930 | | 0.321 ± 0.049 |
| Mouse vas deferens | 4 | 638 ± 190 | | 376 ± 120 | 1.87 ± 0.41 |
| Rat vas deferens | 4 | 15.6± 2.5 | 8.84± 2.2 | 3.29± 4.3 | 0.455± 0.035 |

Each value of both IC50 and relative potency represents the mean±S.E. <sup>a</sup>Number of experiments. <sup>b</sup>IC50 of ME/IC50 of either LE or β-End.

Table 2. Effects of peptidase inhibitors on the inhibitory potency of [Met⁵]-enkephalin in isolated preparations

| Preparations   | N<sup>a</sup> | IC50 (nM) | Relative potency<sup>b</sup> |
|---------------|--------------|-----------|-----------------------------|
|               | Without inhibitors | With inhibitors |                        |
| Guinea-pig ileum | 4 | 380 ± 85 | 54.9 ± 6.5 | 6.79± 1.2 |
| Mouse vas deferens | 4 | 17.8± 3.1 | 0.840± 0.065 | 20.9 ± 2.5 |
| Rat vas deferens | 4 | 55900 ±7000 | 1120 ±170 | 56.3 ±15 |

The IC50 values were estimated in the presence or the absence of three peptidase inhibitors, amastatin, captopril and phosphoramidon, at the final concentration of 1 μM each. Each value of both IC50 and relative potency represents the mean±S.E. <sup>a</sup>Number of experiments. <sup>b</sup>IC50 without inhibitors/IC50 with inhibitors.

Table 3. Effects of peptidase inhibitors on the inhibitory potency of [Leu⁵]-enkephalin in isolated preparations

| Preparations   | N<sup>a</sup> | IC50 (nM) | Relative potency<sup>b</sup> |
|---------------|--------------|-----------|-----------------------------|
|               | Without inhibitors | With inhibitors |                        |
| Guinea-pig ileum | 8 | 1580 ± 660 | 191 ± 50 | 7.66±1.7 |
| Mouse vas deferens | 4 | 10.9± 0.78 | 1.74± 0.033 | 6.30±0.5 |
| Rat vas deferens | 5 | 124000 ±65000 | 2440 ±740 | 44.8 ±8.8 |

The IC50 values were estimated in the presence or the absence of three peptidase inhibitors, amastatin, captopril and phosphoramidon, at the final concentration of 1 μM each. Each value of both IC50 and relative potency represents the mean±S.E. <sup>a</sup>Number of experiments. <sup>b</sup>IC50 without inhibitors/IC50 with inhibitors.

The results obtained in the present study were essentially the same as those reported previously (1–6). The sensitivity against opioid peptides of three kinds of isolated preparations employed in the present study, therefore, was confirmed to be essentially the same as that used in the previous studies (1–6).

Effect of peptidase inhibitors on the potency of opioid peptides: Previous studies (8, 9) had shown that the enkephalin-hydrolyzing aminopeptidase, peptidyl dipeptidase A, and endopeptidase-24.11 were inhibited maximally with either 100 μM of bestatin or 1 μM of amastatin, 1 μM of...
captopril, and either 1 μM of thiorphan or 1 μM of phosphoramidon, respectively.

In the present study, amastatin was employed instead of bestatin as an inhibitor of enkephalin-hydrolyzing aminopeptidase since amastatin was more potent and more soluble in water than bestatin. Additionally, phosphoramidon was employed instead of thiorphan as an inhibitor of enkephalin-hydrolyzing endopeptidase-24.11 in the present investigation since phosphoramidon was more stable, more selective (12) and more soluble in water than thiorphan. Since captopril, an inhibitor of the enkephalin-hydrolyzing peptidyl dipeptidase A, was also soluble in water, the water solution which contained three critical inhibitors of enkephalin-hydrolyzing peptidases could be made easily. The mixture of amastatin, captopril and phosphoramidon at the concentration of 1 mM each was frequently employed in the present study because of its convenience.

The inhibitory potency of both [Met5]-enkephalin and [Leu5]-enkephalin was significantly enhanced by the pretreatment of the isolated preparations with the mixture of three peptidase inhibitors, amastatin, captopril and phosphoramidon. The magnitude of the enhancement by the mixture of the inhibitory potency of [Met5]-enkephalin was the greatest in rat vas deferens, the least in guinea-pig ileum, and intermediate in mouse vas deferens (Table 2), while that of [Leu5]-enkephalin in guinea-pig ileum was similar to that in mouse vas deferens and several-fold less than that in rat vas deferens (Table 3). On the other hand, the pretreatment with the mixture of peptidase inhibitors did not significantly alter the inhibitory potency of β-endorphin in both guinea-pig ileum and rat vas deferens, but significantly augmented the potency of β-endorphin in mouse vas deferens (Table 4). The observation that the potency of enkephalins relative to that of β-endorphin in rat vas deferens was significantly lower than that in the other two preparations in the absence of peptidase inhibitors, therefore, was shown to be partly caused by the presence of the higher activity of enkephalin-hydrolyzing peptidases in rat vas deferens than in the other two preparations. However, even in the presence of the mixture of peptidase inhibitors, like in the absence of inhibitors, the inhibitory potency of both [Met5]- and [Leu5]-enkephalin was the highest in mouse vas deferens, the lowest in rat vas deferens, and intermediate in guinea-pig ileum (Tables 2 and 3), while the potency of β-endorphin was the highest in rat vas deferens, the lowest in guinea-pig ileum, and intermediate in mouse vas deferens (Table 4).

The IC50 values were estimated in the presence or the absence of three peptidase inhibitors, amastatin, captopril and phosphoramidon, at the final concentration of 1 μM each. Each value of both IC50 and relative potency represents the mean±S.E. *Number of experiments. †IC50 without inhibitors/IC50 with inhibitors.

| Preparations         | N | IC50 (nM) Without inhibitors | IC50 (nM) With inhibitors | Relative potency† |
|----------------------|---|----------------------------|--------------------------|-------------------|
| Guinea-pig ileum     | 8 | 154 ± 31                   | 158 ± 31                 | 1.00±0.059        |
| Mouse vas deferens   | 7 | 59.8± 5.1                  | 33.8± 2.7                | 1.78±0.073        |
| Rat vas deferens     | 4 | 11.0± 0.81                 | 10.8± 1.3                | 1.04±0.084        |

The IC50 values of antagonists against opioid peptides: Since β-endorphin was shown not to be inactivated significantly by the enkephalin-hydrolyzing peptidases in both guinea-pig ileum and rat vas deferens, the IC50 values of antagonists against β-endorphin in both preparations were estimated in the absence of the peptidase inhibitor, while those in mouse vas deferens and those against enkephalins in three preparations were determined in the presence of the mixture of three peptidase inhibitors, amastatin, captopril and phosphoramidon. Naloxone had been shown to have a high affinity to mu-receptors (Ks values ranging from 1 to 5 nM) and relatively low affinity to

The Ks values of antagonists against opioid peptides: Since β-endorphin was shown not to be inactivated significantly by the enkephalin-hydrolyzing peptidases in both guinea-pig ileum and rat vas deferens, the Ks values of antagonists against β-endorphin in both preparations were estimated in the absence of the peptidase inhibitor, while those in mouse vas deferens and those against enkephalins in three preparations were determined in the presence of the mixture of three peptidase inhibitors, amastatin, captopril and phosphoramidon. Naloxone had been shown to have a high affinity to mu-receptors (Ks values ranging from 1 to 5 nM) and relatively low affinity to
both kappa- and delta-receptors (Kₑ values ranging from 10 to 50 nM) (3, 10, 13), while Mr 2266 had high affinity to both mu- and kappa-receptors (Kₑ values ranging from 1 to 5 nM) and relatively low affinity to delta-receptors (Kₑ values ranging from 10 to 50 nM) (10, 13). Additionally, ICI 154129 had been reported to have a relatively high affinity to delta-receptors (Kₑ values ranging from 0.2 to 0.9 μM), the lowest affinity to kappa-receptors (Kₑ values being more than 20 μM) and intermediate affinity to mu-receptors (Kₑ values ranging from 7 to 8 μM) (14, 15).

Thus, the Kₑ value of either naloxone or Mr 2266 against either [Met⁵]-enkephalin, [Leu⁵]-enkephalin or β-endorphin (Tables 5, 6 and 7) indicated that these three opioid peptides acted on mu-receptors in guinea-pig ileum and on delta-receptors in mouse vas deferens. Additionally, the Kₑ value of ICI 154129 against both [Met⁵]- and [Leu⁵]-enkephalin (Tables 5 and 6) also suggested that enkephalins acted on mu-receptors in

| Table 5. The Kₑ values of naloxone, Mr 2266 and ICI 154129 against [Met⁵]-enkephalin in isolated preparations |
|--------------------------------------------------|------------------|------------------|------------------|
| Preparations                                   | Nᵃ and Kₑ (nM)   |
|                                                 | Nᵃ   | Nᵃ   | Nᵃ   |
|                                                 | Naloxone | Mr 2266 | ICI 154129 |
| Guinea-pig ileum                               | 4    | 2.76±0.18 | 4    | 1.81±0.11 | 4    | 6430±320 |
| Mouse vas deferens                             | 4    | 33.4±1.4  | 4    | 22.9±2.1  | 4    | 220±9.3  |
| Rat vas deferens                               | 7    | 15.4±3.0  | 7    | 15.3±3.4  | 4    | 2980±850 |

The Kₑ values were estimated in the presence of three peptidase inhibitors, amastatin, captopril and phosphoramidon, at the final concentration of 1 μM each. Each Kₑ value represents the mean±S.E. *Number of experiments.

| Table 6. The Kₑ values of naloxone, Mr 2266 and ICI 154129 against [Leu⁵]-enkephalin in isolated preparations |
|--------------------------------------------------|------------------|------------------|------------------|
| Preparations                                   | Nᵃ and Kₑ (nM)   |
|                                                 | Nᵃ   | Nᵃ   | Nᵃ   |
|                                                 | Naloxone | Mr 2266 | ICI 154129 |
| Guinea-pig ileum                               | 9    | 3.25±0.43 | 9    | 3.41±0.61 | 4    | 7760±1400 |
| Mouse vas deferens                             | 4    | 23.8±2.9  | 4    | 11.7±0.95 | 5    | 122±12    |
| Rat vas deferens                               | 5    | 11.4±1.4  | 5    | 14.2±3.6  | 4    | 3270±940 |

The Kₑ values were estimated in the presence of three peptidase inhibitors, amastatin, captopril and phosphoramidon, at the final concentration of 1 μM each. Each Kₑ value represents the mean±S.E. *Number of experiments.

| Table 7. The Kₑ values of naloxone, Mr 2266 and ICI 154129 against β-endorphin in isolated preparations |
|--------------------------------------------------|------------------|------------------|------------------|
| Preparations                                   | Nᵃ and Kₑ (nM)   |
|                                                 | Nᵃ   | Nᵃ   | Nᵃ   |
|                                                 | Naloxone | Mr 2266 | ICI 154129 |
| Guinea-pig ileum                               | 4    | 3.86±0.39 | 4    | 3.61±0.82 | 4    | 20700±7150 |
| Mouse vas deferens                             | 4    | 31.0±4.2  | 4    | 27.8±2.3  | 4    | 304±25    |
| Rat vas deferens                               | 7    | 10.7±2.1  | 5    | 8.88±1.8  | 4    | 11000±2400 |

The Kₑ values in mouse vas deferens were estimated in the presence of three peptidase inhibitors, amastatin, captopril and phosphoramidon, at the final concentration of 1 μM each, while those in both guinea-pig ileum and rat vas deferens were determined in the absence of the peptidase inhibitor. Each Kₑ value represents the mean±S.E. *Number of experiments.
guinea-pig ileum and on delta-receptors in mouse vas deferens, although both the $K_e$ value of ICI 154129 against [Met$_5$]-enkephalin in guinea-pig ileum and that against [Leu$_5$]-enkephalin in mouse vas deferens (Tables 5 and 6) were slightly lower than those reported previously (14, 15). Although the $K_e$ values in guinea-pig ileum against $\beta$-endorphin of both naloxone and Mr 2266 (Table 7) suggested that $\beta$-endorphin acted on mu-receptors, those of ICI 154129 (Table 7) indicated that $\beta$-endorphin acted on kappa-receptors. The reason for the discrepancy remained to be elucidated.

The $K_e$ value of either naloxone or Mr 2266 against $\beta$-endorphin in rat vas deferens was higher than that in guinea-pig ileum, but lower than that in mouse vas deferens (Table 7), indicating that the $K_e$ value of either naloxone or Mr 2266 against an epsilon agonist was higher than that against a mu agonist, but lower than that against a delta agonist. However, since the $K_e$ value of either naloxone or Mr 2266 against an epsilon agonist was close to the previously reported lowest range of that against a delta agonist, it was difficult to determine whether or not the test compound acted on epsilon receptors by the $K_e$ value of either naloxone or Mr 2266 against the test compound. Therefore, it was difficult to determine whether or not both [Met$_5$]- and [Leu$_5$]-enkephalin acted on epsilon receptors in rat vas deferens by the $K_e$ value of either naloxone or Mr 2266 (Tables 5 and 6). When the $K_e$ value of ICI 154129 against $\beta$-endorphin in rat vas deferens (Table 7) was compared to the previously reported $K_e$ value against either a mu, kappa or delta agonist, it was higher than that against a delta agonist, lower than that against a kappa agonist and similar to or slightly higher than that against a mu agonist. On the other hand, the $K_e$ value of ICI 154129 against both [Met$_5$]- and [Leu$_5$]-enkephalin in rat vas deferens was higher than that against a delta agonist, but lower than that against a mu agonist (Tables 5 and 6). Thus, it was difficult to determine whether or not enkephalins acted on epsilon receptors in rat vas deferens by the $K_e$ value of ICI 154129.

### Table 8. The potency of [Leu$_5$]-enkephalin (LE) or $\beta$-endorphin ($\beta$-End) relative to that of [Met$_5$]-enkephalin (ME) in isolated preparations in the presence of peptidase inhibitors

| Preparations       | IC50 (nM) ME | IC50 (nM) LE | IC50 (nM) $\beta$-End | Relative potency$^b$ |
|--------------------|--------------|--------------|------------------------|----------------------|
| Guinea-pig ileum   | 30.6 ± 5.3   | 101 ± 19     | 36.4 ± 10              | 0.308 ± 0.016        |
| Mouse vas deferens | 1.14± 0.052  | 1.55± 0.085  | 1.16± 0.15             | 0.743 ± 0.032        |
| Rat vas deferens   | 1690 ± 240   | 5920 ± 1100  | 1420 ± 250             | 23.3± 2.1            |

All experiments were carried out in the presence of the mixture of three peptidase inhibitors, amastatin, captopril and phosphoramidon, at the final concentration of 1 $\mu$M each. Each value of both IC50 and relative potency represents the mean±S.E. $^a$Number of experiments. $^b$IC50 of ME/IC50 of either LE or $\beta$-End.

The relative potency of opioid peptides in isolated preparations pretreated with peptidase inhibitors: The potency of [Leu$_5$]-enkephalin or $\beta$-endorphin relative to that of [Met$_5$]-enkephalin was investigated in isolated preparations pretreated with the mixture of peptidase inhibitors, amastatin, captopril and phosphoramidon. The results are shown in Table 8. [Met$_5$]-Enkephalin was approximately three-fold more potent than [Leu$_5$]-enkephalin and four-fold more potent than $\beta$-endorphin in guinea-pig ileum in which three opioid peptides had been indicated to act on mu-receptors (Tables 5, 6 and 7). On the other hand, [Met$_5$]-enkephalin was slightly but significantly more potent than [Leu$_5$]-enkephalin and approximately twenty-fold more potent than $\beta$-endorphin in mouse vas deferens in which
three opioid peptides had been shown to act on delta-receptors (Tables 5, 6 and 7). In rat vas deferens, [Met]$^5$-enkephalin was approximately three-fold more potent than [Leu]$^5$-enkephalin, but sixty-fold less potent than $\beta$-endorphin.

Discussion

It is well-known that [Met]$^5$-enkephalin is more potent than [Leu]$^5$-enkephalin at mu-receptor sites in guinea-pig ileum, while the latter is more potent than the former at delta-receptor sites in mouse vas deferens when the enkephalin-hydrolyzing peptidases are not inhibited (1, 2). The present study, however, shows that [Met]$^5$-enkephalin is more potent than [Leu]$^5$-enkephalin at delta-receptor sites in mouse vas deferens as well as at mu-receptor sites in guinea-pig ileum after the administration of the mixture of the peptidase inhibitors. Since by the administration of the mixture of the peptidase inhibitors, the magnitude of the enhancement of the potency of [Met]$^5$-enkephalin is significantly higher than that of [Leu]$^5$-enkephalin in mouse vas deferens, the observation that [Met]$^5$-enkephalin is less potent than [Leu]$^5$-enkephalin in the absence of the peptidase inhibitors may be caused by the fact that the former is a better substrate for the enkephalin-hydrolyzing enzymes in mouse vas deferens than the latter.

In contrast to enkephalins, $\beta$-endorphin is indicated not to be inactivated in both guinea-pig ileum and rat vas deferens by the enkephalin-hydrolyzing peptidases since the inhibitory potency of $\beta$-endorphin in both guinea-pig ileum and rat vas deferens is not enhanced by the pretreatment of the preparation with the mixture of peptidase inhibitors. The enkephalin-hydrolyzing enzymes in mouse vas deferens, however, are suggested to inactivate $\beta$-endorphin since the inhibitory potency of $\beta$-endorphin in mouse vas deferens is significantly augmented by the pretreatment of mouse vas deferens with the mixture of peptidase inhibitors. Since three enzymes, amastatin-sensitive aminopeptidase, captopril-sensitive peptidyl dipeptidase A, and phosphoramidon-sensitive endopeptidase-24.11 are shown to be all involved in the inactivation of $\beta$-endorphin in mouse vas deferens (manuscript in preparation) and the total activity of three enkephalin-hydrolyzing enzymes in mouse vas deferens is shown to be significantly lower than that in either rat vas deferens or guinea-pig ileum in the present study, the interesting fact that $\beta$-endorphin is not inactivated by enkephalin-hydrolyzing peptidases in both guinea-pig ileum and rat vas deferens but inactivated by those in mouse vas deferens may be caused by some structural differences among enzymes in mouse vas deferens, rat vas deferens and guinea-pig ileum. In fact, the structure of phosphoramidon-sensitive endopeptidase-24.11, an enkephalin-hydrolyzing peptidase, in the kidney has been reported to be slightly different from either that in the intestine (16) or that in the brain (17). Additionally, the report (18) that the lung and brain peptidyl dipeptidases differ markedly in their substrate preference with the lung enzyme, failing to degrade substance K, while the brain enzyme displays similar activity toward substance K and substance P, also indicates the presence of some structural differences between the enzyme in lung and brain. The precise cause of the interesting fact, however, remains to be elucidated in the present study.

Although all possible hydrolysis products of enkephalins have been reported to have very weak or no agonist activity on opioid receptors (19), the hydrolysis products of $\beta$-endorphin having a sequence of [Met]$^5$-enkephalin at their N-terminal have been shown to have significant agonist activity on opioid receptors (6). Thus, the $\beta$-endorphin hydrolysis occurring at any peptide bonds after a sequence of [Met]$^5$-enkephalin can not be estimated by the method employed in the present study. The fact that three enzymes are all involved in the inactivation of $\beta$-endorphin, therefore, indicates that the inactivation of $\beta$-endorphin in mouse vas deferens is most likely to be caused by the cleavage both at the Tyr$^1$-Gly$^2$ bond catalyzed by the amastatin-sensitive aminopeptidase and at the Gly$^3$-Phe$^4$ bond produced by either the captopril-sensitive peptidyl dipeptidase A or the phosphoramidon-sensitive endopeptidase-24.11.

N-Acetylation has been suggested to be
involved in the physiological inactivation of \( \beta \)-endorphin since the presence of \( \beta \)-endorphin acetyltransferase in both brain and pituitary gland has been reported (20), and the acetylated \( \beta \)-endorphin has been shown not to bind to opioid receptors (21). The present study, however, suggests that three enkephalin-hydrolyzing peptidases may be additional enzymes responsible for the physiological inactivation of \( \beta \)-endorphin since these enzymes in mouse vas deferens have been indicated to be located very close to opioid receptors in the previous study (9).

The fact that the inhibitory potency of [Met\(^5\)]-enkephalin relative to that of [Leu\(^5\)]-enkephalin in rat vas deferens is quite similar to that in guinea-pig ileum in which both enkephalins have been shown to act on mu-receptors by the \( K_e \) values indicates that both enkephalins act on mu-receptors in rat vas deferens. However, the observation that the \( K_e \) values of both naloxone and Mr 2266 against either [Met\(^5\)]- or [Leu\(^5\)]-enkephalin in rat vas deferens is different from those against mu-agonists but similar to those against delta-agonists suggests that enkephalins do not act on mu-receptors, but interact with delta-receptors in rat vas deferens. However, the evidence that the \( K_e \) value of ICI 154129 against either [Met\(^5\)]- or [Leu\(^5\)]-enkephalin in rat vas deferens is different from that against delta-agonists indicates that enkephalins do not act on delta-receptors in rat vas deferens. Additionally, the fact that the inhibitory potency of [Met\(^5\)]-enkephalin relative to that of [Leu\(^5\)]-enkephalin in rat vas deferens is different from that in mouse vas deferens in which both enkephalins have been shown to act on delta-receptors by the \( K_e \) values also suggests that both enkephalins do not act on delta-receptors in rat vas deferens. Moreover, the fact that the \( K_e \) value of ICI 154129 in rat vas deferens against \( \beta \)-endorphin, a representative epsilon-agonist, is significantly higher than that against either [Met\(^5\)]- or [Leu\(^5\)]-enkephalin indicates that both enkephalins are likely not to act on epsilon-receptors as well. Thus, the relation between the agonist activity of enkephalin and the type of the opioid receptor in rat vas deferens remains to be elucidated in the present investigation.

References

1. Waterfield, A.A., Smokcum, R.W.J., Hughes, J., Kosterlitz, H.W. and Henderson, G.: In vitro pharmacology of the opioid peptides, enkephalins and endorphins. Eur. J. Pharmacol. 43, 107–116 (1977)
2. Wüster, M., Schulz, R. and Herz, A.: Specificity of opioids towards the \( \mu \)-, \( \delta \)- and \( \epsilon \)-opioid receptors. Neurosci. Lett. 15, 193–198 (1978)
3. Lord, J.A.H., Waterfield, A.A., Hughes, J. and Kosterlitz, H.W.: Endogenous opioid peptides: multiple agonists and receptors. Nature 267, 495–499 (1977)
4. Waterfield, A.A., Leslie, F.M., Lord, J.A.H., Ling, N. and Kosterlitz, H.W.: Opioid activities of fragments of \( \beta \)-endorphin and of its leucine\(^6\)-analog. Comparison of the binding properties of methionine- and leucine-enkephalin. Eur. J. Pharmacol. 58, 11–18 (1979)
5. Lemaire, S., Magnan, J. and Regoli, D.: Rat vas deferens: a specific bioassay for endogenous opioid peptides. Br. J. Pharmacol. 64, 327–329 (1978)
6. Schulz, R., Wüster, M. and Herz, A.: Pharmacological characterization of the \( \epsilon \)-opioid receptor. J. Pharmacol. Exp. Ther. 216, 604–606 (1981)
7. McKnight, A.T., Corbett, A.D. and Kosterlitz, H.W.: Increase in potencies of opioid peptides after peptidase inhibition. Eur. J. Pharmacol. 86, 393–402 (1983)
8. Aoki, K., Kajiwara, M. and Oka, T.: The role of bestatin-sensitive aminopeptidase, angiotensin converting enzyme and thiorphan-sensitive "enkephalinase" in the potency of enkephalins in the guinea-pig ileum. Japan. J. Pharmacol. 36, 59–65 (1984)
9. Aoki, K., Kajiwara, M. and Oka, T.: The inactivation of [Met\(^6\)]-enkephalin by bestatin-sensitive aminopeptidase, captopril-sensitive peptidyl dipeptidase A and thiorphan-sensitive endopeptidase-24.11 in mouse vas deferens. Japan. J. Pharmacol. 40, 297–302 (1986)
10. Oka, T., Negishi, K., Suda, M., Sawa, A., Fujino, M. and Wakimasu, M.: Evidence that dynorphin (1–13) acts as an agonist on opioid \( \alpha \)-receptors. Eur. J. Pharmacol. 77, 137–141 (1982)
11. Kosterlitz, H.W. and Watt, A.J.: Kinetic parameters of narcotic agonists and antagonists, with particular reference to N-allylnoroxymorphone (naloxone). Br. J. Pharmacol. 33, 266–276 (1968)
12. Matsas, R., Kenny, A.J. and Turner, A.J.: The
metabolism of neuropeptides: the hydrolysis of peptides, including enkephalins, tachykinins and their analogues, by endopeptidase-24.11. Biochem. J. 224, 433–440 (1984)

13 Oka, T., Negishi, K., Kajiwara, M., Watanabe, Y., Ishizuka, Y. and Matsumiya, T.: The choice of opiate receptor subtype by neo-endorphins. Eur. J. Pharmacol. 79, 301–305 (1982)

14 Shaw, J.S., Miller, L., Turnbull, M.J., Gormley, J.J. and Morley, J.S.: Selective antagonists at the opiate delta-receptor. Life Sci. 31, 1259–1262 (1982)

15 Ueki, M., Aoki, K., Kajiwara, M., Shinozaki, K., Inoue, H. and Oka, T.: Two new opioid delta-receptor ligands: a highly selective agonist and a potent selective antagonist in in vitro isolated preparations. Japan. J. Pharmacol. 36, 485–489 (1984)

16 Fulcher, I.S., Chaplin, M.F. and Kenny, A.J.: Endopeptidase-24.11 purified from pig intestine is differentially glycosylated from that in kidney. Biochem. J. 215, 317–323 (1983)

17 Relton, J.M., Gee, N.S., Matsas, R., Turner, A.J. and Kenny, A.J.: Purification of endopeptidase-24.11 ("enkephalinase") from pig brain by immunoadsorbent chromatography. Biochem. J. 215, 519–523 (1983)

18 Thiele, E.A., Strittmatter, S.M. and Snyder, S.H.: Substance K and substance P as possible endogenous substrates of angiotensin converting enzyme in the brain. Biochem. Biophys. Res. Commun. 128, 317–324 (1985)

19 Morley, J.S.: Structure-activity relationships of enkephalin-like peptides. Annu. Rev. Pharmacol. Toxicol. 20, 81–110 (1980)

20 O’Donohue, T.L.: Identification of endorphin acetyltransferase in rat brain and pituitary gland. J. Biol. Chem. 258, 2163–2167 (1983)

21 Smyth, D.G., Massey, D.E., Zakarian, S. and Finnie, M.D.: Endorphins are stored in biologically active and inactive forms: isolation of α-N-acetyl peptides. Nature 279, 252–254 (1979)