The Role of Bestatin-Sensitive Aminopeptidase, Angiotensin Converting Enzyme and Thiorphan-Sensitive “Enkephalinase” in the Potency of Enkephalins in the Guinea-Pig Ileum

Kazuko AOKI, Midori KAJIWARA and Tetsuo OKA*
Department of Pharmacology, School of Medicine, Tokai University, Isehara 259-11, Japan
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Abstract—The role of each enkephalin-hydrolyzing peptidase in the inhibitory potency of exogenously added enkephalins in the myenteric plexus-longitudinal muscle preparation of guinea-pig ileum was studied by using the relatively specific inhibitor of each enzyme. Results showed that three distinct enzymes, bestatin-sensitive aminopeptidase(s), angiotensin converting enzyme, and thiorphan-sensitive “enkephalinase”, played a critical role in the inactivation of enkephalins. Additionally, these enzymes are likely to be located close to opioid receptors, since they produce a significant concentration difference of enkephalin between the surrounding organ bath and the vicinity of opioid receptors. In contrast to these three enzymes, both L-tyrosyl-L-tyrosine-sensitive dipeptidyl aminopeptidase and D-phenylalanine-sensitive carboxypeptidase are indicated not to be involved significantly in the degradation of exogenously added enkephalins in the guinea-pig ileum.

It has already been reported that peptidase inhibitors enhance the inhibitory potency of exogenously given enkephalin in an in vitro isolated preparation of guinea-pig ileum (1–5). However, controversy exists over the augmentative effectiveness of peptidase inhibitors against the inhibitory potency of enkephalin in guinea-pig ileum. The antibiotic puromycin, which had been shown to be an effective inhibitor of the hydrolysis of enkephalin by brain homogenate (6), was reported to prolong greatly the depressant effect of enkephalin on the electrically-induced contractions of guinea-pig ileum (1). However, Cohen et al. (2) showed that the inhibitory potency of enkephalin in guinea-pig ileum was not enhanced by puromycin, but augmented by bestatin, an aminopeptidase inhibitor of bacterial origin (7); and the degradation of $^3$H-enkephalin after incubation with guinea-pig ileum was not altered with puromycin, but decreased with bestatin. On the other hand, it was reported (3) that the inhibitory potency of enkephalin in guinea-pig ileum was not altered with either thiorphan, an inhibitor of “enkephalinase” (8), or captopril, an angiotensin converting enzyme inhibitor (9). However, Kosterlitz and his colleague (4, 5) showed that the greatest increase in potency of enkephalin in guinea-pig ileum was obtained with the combination of bestatin, captopril, thiorphan and L-leucyl-L-leucine.

Figure 1 illustrates the possible sites of $[\text{Met}^5]$-enkephalin hydrolysis, peptidase activities involved, and peptidase inhibitors employed in the present investigation. The role of each peptidase in the enkephalin hydrolysis in guinea-pig ileum was studied in the present investigation by using the relatively specific inhibitor of each peptidase.

Materials and Methods

Chemicals: Gifts of compounds which were gratefully received were normorphine-HCl from Dr. T. Muraki, Keio University

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* To whom all correspondence should be addressed.
(Tokyo), bestatin from Nippon Kayaku (Tokyo), captopril from Sankyo Company (Tokyo), and thiorphan from Dr. M. Nozaki, Gifu University (Gifu). [Met$_5$]-Enkephalin, [Leu$_5$]-enkephalin, amastatin and D-phenylalanine were purchased from Peptide Institute, Inc. (Minoh), puromycin-2HCl from Makor Chemicals, Ltd. (Jerusalem, Israel), and L-tyrosyl-L-tyrosine from Miles-Yada, Ltd. (Israel).

In vitro isolated preparations: Male Hartley guinea-pigs weighing 300–500 g were used for this study. The myenteric plexus-longitudinal muscle strip of guinea-pig ileum was prepared and set up for electrical stimulation as described previously (11). The % inhibition of the stimulated muscle twitch produced by a drug was plotted against the log concentration of the drug to estimate the IC$_{50}$ (concentration of the drug to produce 50% inhibition of the twitch). When the influence of drugs like bestatin, captopril or thiorphan on the enkephalin-induced inhibition of contractions was studied, peptidase inhibitors were given at least three minutes before the enkephalin administration; The addition of a peptidase inhibitor significantly enhanced the enkephalin-induced inhibition of contractions, indicating that a peptidase may cause the hydrolysis of exogenously added enkephalin in the guinea-pig ileum since all possible hydrolysis products of enkephalin have been reported to have very weak or no agonist activity in the guinea-pig ileum (12). The significance of % differences between IC$_{50}$ values of two groups was determined by Student’s $t$-test.

Results

The control experiment: The IC$_{50}$ value of normorphine was repeatedly estimated in seven preparations of guinea-pig ileum for six hours. The change in the IC$_{50}$ value of normorphine with the passage of time was quite small in all preparations (Table 1), showing that the response of the preparation to an opioid was not significantly changed during the experiment. The IC$_{50}$ value of [Met$_5$]-enkephalin was also repeatedly determined in four preparations for five hours. The IC$_{50}$ value of [Met$_5$]-enkephalin in all preparations was not changed significantly with the passage of time (Table 1), indicating that the activity of enkephalin-hydrolyzing peptidases in the preparation was not significantly altered during the experiment.

Effects of aminopeptidase inhibitors on the inhibitory potency of enkephalins: The inhibitory effect of enkephalins on the electrically-evoked contractions of guinea-pig ileum was significantly enhanced by the pretreatment of the preparation with aminopeptidase inhibitors such as bestatin and amastatin (Tables 2 and 3). The enhancing effect of amastatin or bestatin on the enkephalin-induced inhibition reached maximum at the dose of 1 or 100 $\mu$M, respectively.

Fig. 1. Possible sites of [Met$_5$]-enkephalin hydrolysis, peptidase activities involved (solid arrows), and peptidase inhibitors employed (dotted arrows). "Enkephalinase" has recently been reported to be identical with endopeptidase 24.11 (10).
In contrast to amastatin and bestatin, puromycin at doses ranging from 1 to 100 μM had no enhancing effect on the inhibitory potency of enkephalins (data are not shown). The IC50 values of [Met5]-enkephalin in preparations pretreated with bestatin (100 μM) alone were not significantly different from those pretreated with both bestatin (100 μM) and amastatin (1 μM) (Table 4), indicating that the aminopeptidase inhibited by bestatin was the same as that depressed by amastatin.

### Table 1. The repeated estimation of the IC50 values of normorphine and [Met5]-enkephalin in guinea-pig ileum

| Order of estimation | Normorphine IC50 (nM) | Ratio of potency | % Difference | IC50 (nM) | Ratio of potency | % Difference |
|---------------------|-----------------------|------------------|--------------|-----------|-----------------|--------------|
| Initial             | 245±42                | 1                |              | 265±54    | 1               | 2.50±3.8     |
| Second              | 247±45                | 1.00±0.01        | 0.14±1.4     | 258±54    | 1.03±0.04       | 3.00±5.5     |
| Third               | 249±45                | 1.01±0.03        | 1.57±3.1     | 278±68    | 0.98±0.06       | 0.25±5.7     |
| Fourth              | 250±48                | 1.01±0.05        | 0.43±4.5     | 273±72    | 1.01±0.06       | 1.50±5.5     |
| Fifth               |                       |                  |              | 262±45    | 1.00±0.05       |              |

Each value represents the mean±S.E. of 7 experiments with normorphine and 4 experiments with [Met5]-enkephalin.

### Table 2. Enhancing effect of bestatin on the inhibitory potency of enkephalin in guinea-pig ileum

| Bestatin (μM) | [Met5]-Enkephalin IC50 (nM) | Ratio of potency | % Difference | [Leu7]-Enkephalin IC50 (nM) | Ratio of potency | % Difference |
|--------------|-----------------------------|------------------|--------------|-----------------------------|------------------|--------------|
| 0            | 216±58                      | 1                |              | 604±150                     | 1                | 75.3±0.85*** |
| 10           | 50.7±13                     | 4.28±0.51        | 75.5±2.7***  | 147±33                      | 4.09±0.15        | 13.3±3.5*    |
| 100          | 35.2±11                     | 6.41±0.85        | 32.3±3.6***  | 129±34                      | 4.71±0.21        | 5.5±3.3      |
| 200          | 35.2±10                     | 6.26±0.68        |              | 134±32                      | 4.47±0.10        |              |

Each value represents the mean±S.E. of 4 experiments. *P<0.05, ***P<0.01.

### Table 3. Enhancing effect of amastatin on the inhibitory potency of [Met5]-enkephalin in guinea-pig ileum

| Amastatin (μM) | IC50 (nM) | Ratio of potency | % Difference |
|---------------|-----------|------------------|--------------|
| 0             | 309±98    | 1                |              |
| 0.1           | 149±37    | 1.96±0.16        | 47.8±4.3***  |
| 1             | 78.6±12   | 3.64±0.68        | 43.5±5.3***  |
| 10            | 80.4±16   | 3.62±0.58        | 1.0±7.4      |

Each value represents the mean±S.E. of 4 experiments. ***P<0.01.
Enhanced effect of captopril on the inhibitory potency of enkephalins: The IC50 values of enkephalins in preparations treated with both bestatin and captopril were significantly lower than those treated with bestatin alone (Table 5), indicating that the angiotensin converting enzyme in preparations hydrolyzed enkephalins and decreased their potencies. The enhancing effect of captopril on the enkephalin-induced inhibition in preparations pretreated with bestatin reached maximum at the dose of 1 μM (Table 5).

Enhancing effect of thiorphan on the inhibitory potency of enkephalins: The IC50 values of enkephalins in preparations treated

| Inhibitors                        | IC50 (nM) | Ratio of potency | % Difference |
|----------------------------------|-----------|------------------|--------------|
| None                             | 222 ± 42  | 1                | 75.9 ± 1.43*** |
| 100 μM Bestatin                  | 52.3 ± 7.7| 4.18±0.24        | 0.068±6.7    |
| 100 μM Bestatin+1 μM amastatin   | 52.5 ± 9.3| 4.19±0.15        |              |

Amastatin was added to the bath immediately after the bestatin administration. ***P<0.01.

Table 5. Enhancing effect of captopril on the inhibitory potency of enkephalin in preparations pretreated with bestatin

| Captopril (μM) | [Met⁵]-Enkephalin | [Leu⁵]-Enkephalin |
|----------------|-------------------|-------------------|
| IC50 (nM)      | Ratio of potency  | % Difference      | IC50 (nM)      | Ratio of potency  | % Difference      |
| 0              | 37.5±1.7          | 1                 | 133 ±5.9       | 1               |                   |
| 0.1            | 30.1±1.1          | 1.25±0.04         | 92.2±2.9       | 1.45±0.08       | 18.5±1.9***       |
| 1              | 26.1±0.56         | 1.44±0.04         | 75.0±0.6       | 1.78±0.08       | 0.25±2.7          |
| 10             | 26.6±1.1          | 1.41±0.02         | 74.9±2.3       | 1.78±0.03       |                   |

All preparations were pretreated with 100 μM of bestatin immediately before the captopril or vehicle administration. Each value represents the mean±S.E. of 4 experiments. **P<0.02, ***P<0.01.

Table 6. Enhancing effect of thiorphan on the inhibitory potency of enkephalin in preparations pretreated with both bestatin and captopril

| Thiorphan (μM) | [Met⁵]-Enkephalin | [Leu⁵]-Enkephalin |
|----------------|-------------------|-------------------|
| IC50 (nM)      | Ratio of potency  | % Difference      | IC50 (nM)      | Ratio of potency  | % Difference      |
| 0              | 28.2±1.3          | 1                 | 74.1±6.8       | 1               |                   |
| 0.1            | 23.3±0.82         | 1.21±0.02         | 61.5±5.4       | 1.20±0.009      | 6.0±1.6*          |
| 1              | 20.8±0.66         | 1.35±0.03         | 57.7±4.3       | 1.28±0.03       | 0.5±1.6           |
| 10             | 20.9±1.0          | 1.35±0.02         | 58.1±5.0       | 1.27±0.02       |                   |

All preparations were pretreated with both 100 μM of bestatin and 1 μM of captopril immediately before the thiorphan or vehicle administration. Each value represents the mean±S.E. of 4 experiments. *P<0.05, ***P<0.01.
with three peptidase inhibitors, bestatin, captopril and thiorphan, were significantly lower than those treated with the former two inhibitors, bestatin and captopril (Table 6). Results indicate that thiorphan inhibits a different peptidase from that inhibited by either bestatin or captopril and increases the potency of enkephalins by preventing their hydrolysis. The enhancing effect of thiorphan on the enkephalin-induced inhibition in preparations pretreated with both bestatin and captopril reached maximum at the dose of 1 nM (Table 6).

Negative effect of L-tyrosyl-L-tyrosine or D-phenylalanine on the inhibitory potency of enkephalins: The IC50 values of enkephalins in preparations treated with both 100 nM of bestatin and 100 nM of L-tyrosyl-L-tyrosine or 100 nM of D-phenylalanine were not significantly different from those treated with 100 nM of bestatin alone (Table 7).

Inhibitory actions of peptidase inhibitors after the [Met⁵]-enkephalin administration: When peptidase inhibitors such as bestatin, captopril and thiorphan were added to the bath after the enkephalin administration, they produced the inhibition of contractions of guinea-pig ileum (Fig. 2), although peptidase inhibitors by themselves did not produce the inhibition of contractions when an opioid peptide was absent in the bath. Since the inhibition of contractions produced by peptidase inhibitors was reversed by naloxone (Fig. 2), the inhibition must be caused by the increased amount of [Met⁵]-enkephalin available for binding sites of opioid receptors.

### Table 7. Negative effect of L-tyrosyl-L-tyrosine or D-phenylalanine on the inhibitory potency of enkephalin in preparations pretreated with bestatin

| Compounds                  | [Met⁵]-Enkephalin | [Leu⁵]-Enkephalin |
|----------------------------|-------------------|-------------------|
|                            | IC50 (nM)         | Ratio of potency | Difference | IC50 (nM) | Ratio of potency | % Difference |
| 100 μM Bestatin (control 1)| 43.8± 2.8         | 1                 |           | 201±23    | 1                 | 1.50±6.1     |
| 100 μM Bestatin +100 μM L-Tyr-L-Tyr | 45.5± 4.7         | 0.99±0.1          | 4.50±11   | 207±34    | 1.02±0.06         |             |
| 100 μM Bestatin (control 2)| 56.3±13           | 1                 |           | 156±22    | 1                 | 3.75±5.1     |
| 100 μM Bestatin+100 μM D-Phe| 53.5±13           | 1.06±0.07         |           | 159±17    | 1.04±0.05         |             |

L-Tyrosyl-L-tyrosine or D-phenylalanine was administered immediately after the bestatin administration. Each value represents the mean±S.E. of 4 experiments.

Discussion

The control experiment in the present investigation shows that although the IC50 value of normorphine or [Met⁵]-enkephalin in one preparation is sometimes significantly different from that in another preparation, the change in the IC50 value of normorphine or [Met⁵]-enkephalin in one preparation with the passage of time is always quite small for at least five hours. The results indicate that the response of the preparation to an opioid, the sensitivity of opioid receptors to an opioid, or the activity of enkephalin-hydrolyzing peptidases in the preparation is not significantly altered during the usual experimental period. Thus, the fact that the
IC50 values of enkephalins are significantly decreased by the pretreatment of the preparation with aminopeptidase inhibitors such as bestatin and amastatin indicates that the aminopeptidase in the preparation of guinea-pig ileum hydrolyzes the exogenously administered enkephalins and decreases their inhibitory potencies in the preparation. The enkephalin-hydrolyzing aminopeptidase was found to be inhibited maximally with either amastatin or bestatin at the dose of 1 or 100 μM, respectively, since the enhanced effect of amastatin or bestatin on the enkephalin-induced inhibition reaches maximum at the dose of 1 or 100 μM, respectively. The fact that the IC50 values of enkephalins in preparations pretreated with bestatin at the dose of 100 μM alone are not significantly different from those pretreated with both 100 μM bestatin and 1 μM amastatin suggests that the enkephalin-hydrolyzing aminopeptidase inhibited by bestatin is the same enzyme as that depressed by amastatin. Since puromycin at doses ranging from 1 to 100 μM has no enhancing effect on the enkephalin-induced inhibition, the enkephalin-hydrolyzing aminopeptidase in guinea-pig ileum is likely not to be significantly inhibited by puromycin.

The involvement of angiotensin converting enzyme in the hydrolysis of exogenously administered enkephalins in guinea-pig ileum is suggested by the fact that the IC50 values of enkephalins in preparations treated with both bestatin and captopril, an angiotensin converting enzyme inhibitor, are significantly lower than those treated with bestatin alone. In addition to aminopeptidase and angiotensin converting enzyme, “enkephalinase” is likely to be also involved in the degradation of exogenously given enkephalins in guinea-pig ileum since the IC50 values of enkephalins in preparations treated with bestatin, captopril and thiorphan, an inhibitor of “enkephalinase”, are significantly lower than those treated with bestatin alone.

In contrast to the enkephalin-hydrolyzing peptidases such as aminopeptidase, angiotensin converting enzyme and “enkephalinase”, both dipeptidyl aminopeptidase and carboxypeptidase seem not to be involved significantly in the metabolism of exogenously added enkephalins in guinea-pig ileum since the IC50 values of enkephalins in the preparations pretreated with bestatin alone are not significantly different from those pretreated with both bestatin and L-tyrosyl-L-tyrosine, which has been shown to be an inhibitor of an amino-terminal-directed dipeptidylpeptidase (13), or D-phenylalanine which has been reported to be a carboxypeptidase inhibitor (14). Additionally, the fact that the degradation of 3H-[Leu5]-enkephalin after incubation with the preparation of guinea-pig ileum is almost completely prevented by the pretreatment of the preparation with bestatin, captopril and thiorphan (manuscript in preparation) indicates that both dipeptidyl aminopeptidase and carboxypeptidase do not play a significant role in the hydrolysis of the enkephalin in the preparation of guinea-pig ileum.

The fact that the enkephalin-hydrolyzing peptidase inhibitors produce the inhibition of contractions of guinea-pig ileum by themselves after the enkephalin administration and the inhibition is reversed by naloxone shows that the organ bath serves as an inexhaustible reservoir of exogenously added enkephalin at least for several minutes, and when the enkephalin-hydrolyzing peptidase inhibitors are absent, the concentration of enkephalin in the vicinity of opioid receptors is significantly lower than that in the surrounding organ bath. Additionally, the fact that the potency of enkephalin in the guinea-pig ileum is not changed significantly by washing the tissue many times for several hours indicates that the enkephalin-hydrolyzing peptidases are not “released” but are membrane-bound ones. Since enkephalin can arrive in the vicinity of opioid receptors from various directions of the surrounding organ bath, it is likely that the enkephalin-hydrolyzing enzymes in the preparation of guinea-pig ileum are located very close to opioid receptors and produce a significant concentration difference of enkephalin between the surrounding organ bath and the vicinity of opioid receptors.

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