The Enhancing Effects of Amastatin, Phosphoramidon and Captopril on the Potency of [Met⁵]-Enkephalin in Rat Vas Deferens

Suying CUI[#], Midori KAJIWARA, Kaori ISHII, Kazuko AOKI,
Junshi SAKAMOTO, Teruhiko MATSUMIYA and Tetsuo OKA*

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

Accepted May 29, 1986

Abstract—The enkephalin-inactivating enzymes in rat vas deferens were studied by using the relatively specific inhibitor of each enzyme. The results showed that the rat vas deferens, like the other three preparations, guinea-pig ileum, mouse vas deferens and striatal membranes of guinea-pig brain, which had been investigated previously, contained three distinct enkephalin-hydrolyzing peptidases. Additionally, the enkephalin-hydrolyzing aminopeptidase, endopeptidase-24.11 and peptidyl dipeptidase A in rat vas deferens were found to be inhibited maximally with 1 μ M of amastatin, 1 μ M of phosphoramidon and 1 μ M of captopril, respectively. In contrast to these three enzymes, both L-tyrosyl-L-tyrosine-sensitive dipeptidyl aminopeptidase and D-phenylalanine-sensitive carboxypeptidase were suggested not to be involved significantly in the inactivation of exogenously given enkephalin in rat vas deferens. The characteristics of the enkephalin-degradative enzymes in rat vas deferens were discussed in terms of their similarities to and differences from those in the other preparations.

Three enzymes, both bestatin- and amastatin-sensitive aminopeptidase, both thiorphan- and phosphoramidon-sensitive endopeptidase-24.11 ("enkephalinase", EC 3.4.24.11), and captopril-sensitive peptidyl dipeptidase A (angiotensin I converting enzyme, kininase II, peptidyl dipeptide hydrolase, EC 3.4.15.1), have been shown to play important roles in the inactivation of exogenously given enkephalin in two in vitro isolated preparations, guinea-pig ileum (1) and mouse vas deferens (2). Additionally, these three enzymes have been indicated to be located very close to opioid receptors (1, 2). Moreover, enkephalin has been shown to be exclusively inactivated by these three

peptidases, since enkephalin remains intact almost totally after it is incubated with either ileal or striatal membrane fractions of guinea-pig for 60 min at 37°C in the presence of three peptidase inhibitors, amastatin, thiorphan and captopril, although free tyrosine and the Tyr-Gly-Gly fragment are produced when the incubation mixture does not contain the peptidase inhibitor (3).

On the other hand, the structure of phosphoramidon-sensitive endopeptidase-24.11 in the kidney has been reported to be slightly different from either that in the intestine (4) or that in the brain (5). Additionally, the lung and brain peptidyl dipeptidases have been shown to 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 (6). Moreover, the inhibitory potency of β -endorphin has been shown to be significantly enhanced by the pretreatment

[#] Present address: Department of Physiology, Institute of Clinical Research, China-Japan Friendship Hospital, Heping North Street, Beijing, China.

* To whom reprint requests should be addressed.

of the mouse vas deferens with three peptidase inhibitors, amastatin, captopril and phosphoramidon, while it is not augmented in both guinea-pig ileum and rat vas deferens by the same pretreatment (7). These four reports suggest that the structure or the function of a particular peptidase in one preparation or tissue may not always be the same as that in the other.

In the present investigation, therefore, the effect of peptidase inhibitor on the potency of [Met⁵]-enkephalin in rat vas deferens was examined in order to elucidate whether or not the enkephalin-hydrolyzing peptidase in rat vas deferens had the same characteristics as that in guinea-pig ileum (1) or mouse vas deferens (2).

Materials and Methods

Chemicals: Gifts of compounds which were gratefully received were amastatin and phosphoramidon from Dr. T. Aoyagi, Institute of Microbial Chemistry (Tokyo), and captopril from Sankyo Company (Tokyo). [Met⁵]-Enkephalin and D-phenylalanine were purchased from Peptide Institute, Inc. (Minoh), and L-tyrosyl-L-tyrosine was from Miles-Yeda, Ltd. (Israel).

In vitro isolated preparations: Male Wistar rats weighing 300–500 g were used for this study. The vasa deferentia from rats were prepared and set up for electrical stimulation as described previously (8). The % inhibition of the stimulated muscle twitch produced by [Met⁵]-enkephalin was plotted against the log concentration of the opioid to estimate the IC₅₀ (concentration of the opioid to produce 50% inhibition of the twitch). When the effect of peptidase inhibitors on the IC₅₀ value of [Met⁵]-enkephalin was studied, they were given at least ten minutes before the enkephalin administration. The % difference shown in the table was calculated as described previously (2). The significance of % differences between IC₅₀ values of two adjacent groups shown in the table was determined by the paired Student's *t*-test.

Results

The control experiment: The IC₅₀ value of [Met⁵]-enkephalin was repeatedly estimated for five times in six preparations of rat vas

deferens in order to examine the stability of the response of the preparation to an opioid during the usual experimental period, since it has been shown that the sensitivity to an opioid of rabbit vas deferens was significantly increased with the passage of time (9), while that of guinea-pig ileum was not significantly changed during the usual experimental period (1). The IC₅₀ values of [Met⁵]-enkephalin estimated initially and secondarily were significantly higher than those estimated secondarily and thirdly, respectively (Table 1). On the other hand, the IC₅₀ values estimated thirdly and fourthly were not significantly different from those estimated fourthly and fifthly, respectively (Table 1). Since the increased sensitivity of the preparation to an opioid during the period from the initial to the third determination of the IC₅₀ value (Table 1) might be caused by the decreased activity of the enkephalin-hydrolyzing enzymes during the identical period, the IC₅₀ value of [Met⁵]-enkephalin was then repeatedly estimated in the presence of three peptidase inhibitors, amastatin, captopril and phosphoramidon, at the final concentration of 1 μ M each. The IC₅₀ values of [Met⁵]-enkephalin in the presence of three peptidase inhibitors were also decreased during the period from their initial to third determinations (Table 1), indicating the negative involvement of the peptidases in the increased sensitivity of the preparation to an opioid during the early phase of the experiment. Since the results obtained in both the absence and the presence of the peptidase inhibitors showed that the response of the preparation to an opioid was not significantly changed during the period from the third to the fifth estimation of the IC₅₀ value, the following experiments on the effect of the peptidase inhibitor on the potency of [Met⁵]-enkephalin in rat vas deferens were carried out during the equivalent period from the third to the fifth estimation of the IC₅₀ value.

Enhanced effect of the peptidase inhibitor on the potency of [Met⁵]-enkephalin: [Met⁵]-Enkephalin had been shown to be hydrolyzed by three distinct enzymes in several preparations such as guinea-pig ileum (1), mouse vas deferens (2), and both

Table 1. The repeated estimation of the IC₅₀ values of [Met⁵]-enkephalin in either the presence or the absence of the mixture of peptidase inhibitors

Order of estimation	[Met ⁵]-Enkephalin without peptidase inhibitor			[Met ⁵]-Enkephalin with peptidase inhibitors ^a		
	IC ₅₀ (μM)	Ratio of potency	% Difference	IC ₅₀ (μM)	Ratio of potency	% Difference
Initial	43.9±9.3	1		1.39 ±0.35	1	
Second	36.7±9.5	1.25±0.044	19.2 ±2.9***	1.09 ±0.22	1.23±0.064	17.5 ±3.9***
Third	30.8±6.1	1.42±0.036	12.2 ±2.7***	0.973±0.20	1.38±0.094	10.6 ±2.7***
Fourth	30.3±7.5	1.53±0.13	4.52 ±6.3	0.932±0.16	1.42±0.13	1.77±2.6
Fifth	29.5±6.3	1.54±0.13	0.433±3.2	0.957±0.18	1.40±0.11	1.57±1.5

The approximate interval of each [Met⁵]-enkephalin administration was 30 min. Each value represents the mean±S.E. of 6 experiments. ^aThe mixture of three peptidase inhibitors, amastatin, captopril and phosphoramidon, was given 10 min before the enkephalin administration at the final concentration of 1 μM each. ***P<0.01.

Table 2. Enhancing effect of amastatin on the inhibitory potency of [Met⁵]-enkephalin in rat vas deferens pretreated with both phosphoramidon and captopril

Amastatin (μM)	IC ₅₀ (nM)	Ratio of potency	% Difference
0.1	613±130	1	
1	257± 36	2.32±0.18	55.8 ± 3.4***
2	241± 42	2.33±0.20	0.884±10

The mixture of two peptidase inhibitors, phosphoramidon and captopril, was added 10 min before the enkephalin administration at the final concentration of 1 μM each. Amastatin was given immediately after the mixture administration. Each value represents the mean±S.E. of 4 experiments. ***P<0.01.

ileal and striatal membrane fractions of guinea-pig (3). Therefore, the presence of the enzyme with low enkephalin-hydrolyzing activity was sometimes difficult to detect when the residual enzyme with high enkephalin-hydrolyzing activity was not inhibited (3). Then, when the enkephalin-hydrolyzing ability of a particular enzyme among three enzymes was examined, the residual two enzymes were inhibited in advance in the present study.

Initially, the effect of amastatin, an aminopeptidase inhibitor, on the inhibitory potency of [Met⁵]-enkephalin in the rat vas deferens pretreated with two peptidase inhibitors, phosphoramidon and captopril, at the final concentration of 1 μM each was examined. The IC₅₀ value of enkephalin in the presence

of 1 μM of amastatin was not significantly different from that of 2 μM of amastatin, but significantly lower than that of 0.1 μM of amastatin (Table 2). This implied that the enkephalin-hydrolyzing aminopeptidase in rat vas deferens was inhibited maximally with amastatin at the final concentration of 1 μM, being consistent with the previous findings obtained with guinea-pig ileum (1) and mouse vas deferens (2). However, Corbett et al. (10) and McKnight et al. (11) have suggested that the susceptibility to bestatin, another aminopeptidase inhibitor, of the enkephalin-hydrolyzing aminopeptidase in rat vas deferens was lower than that in guinea-pig ileum or mouse vas deferens, since they had used 30 μM of bestatin in rat vas deferens but 10 μM of bestatin in both

guinea-pig ileum and mouse vas deferens to inhibit the enkephalin-hydrolyzing aminopeptidase. In order to confirm the present observation, therefore, the effect of the increasing doses of amastatin on the electrically-evoked contractions of the rat vas deferens pretreated with both captopril and phosphoramidon was examined in the presence of [Met⁵]-enkephalin in the organ bath. Amastatin at the final concentration of 0.1 μ M significantly enhanced the enkephalin-induced inhibition of the contractions of the rat vas deferens pretreated with both captopril and phosphoramidon (Fig. 1). Additionally, the enhanced inhibition produced by 0.1 μ M of amastatin was further augmented by increasing the dose of amastatin to 1 μ M (Fig. 1). However, the inhibition induced by 1 μ M of amastatin was not further enhanced

Fig. 1. Naloxone (Nal)-reversible inhibitory action of amastatin (Ama) on electrically-evoked contractions of rat vas deferens in the presence of [Met⁵]-enkephalin (ME). The mixture of captopril and phosphoramidon at the final concentration of 1 μ M each was given 10 min before the enkephalin administration. Compounds were added to the bath at the dot. Note that amastatin at the final concentration of 10⁻⁷ M enhances, and that of 10⁻⁶ M further enhances, but that of 10⁻⁵ M no more augments the inhibition of contractions.

by increasing the dose of amastatin to 10 μ M (Fig. 1). The inhibition produced by the administration of both [Met⁵]-enkephalin and amastatin was completely reversed by 1 μ M of naloxone, a specific opioid antagonist (Fig. 1). The result suggested that the enkephalin-hydrolyzing aminopeptidase was inhibited maximally with amastatin at the final concentration of 1 μ M, and the amastatin-induced inhibition of the contractions of the rat vas deferens in the presence of enkephalin was caused by the increased amount of an opioid in the vicinity of receptors.

Secondarily, the effect of phosphoramidon, an inhibitor of endopeptidase-24.11, on the inhibitory potency of [Met⁵]-enkephalin in the rat vas deferens pretreated with two peptidase inhibitors, amastatin and captopril, at the final concentration of 1 μ M each was estimated. The IC₅₀ value of enkephalin in the presence of 1 μ M of phosphoramidon was not significantly different from that of 2 μ M of phosphoramidon, but significantly lower than that of 0.1 μ M of phosphoramidon (Table 3). This indicated that the enkephalin-hydrolyzing endopeptidase-24.11 in rat vas deferens was inhibited maximally with phosphoramidon at the final concentration of 1 μ M, being consistent with the previous results obtained with guinea-pig ileum (1), mouse vas deferens (2), pig caudate synaptic membranes (12) and purified endopeptidase-24.11 from pig kidney (13).

Thirdly, the effect of captopril, an inhibitor of peptidyl dipeptidase A, on the inhibitory potency of [Met⁵]-enkephalin in the rat vas deferens pretreated with two peptidase inhibitors, amastatin, and phosphoramidon, at the final concentration of 1 μ M each was

Table 3. Enhancing effect of phosphoramidon on the inhibitory potency of [Met⁵]-enkephalin in rat vas deferens pretreated with both amastatin and captopril

Phosphoramidon (μ M)	IC ₅₀ (μ M)	Ratio of potency	% Difference
0.1	4.12 $\pm$ 3.1	1	
1	1.96 $\pm$ 1.3	1.72 $\pm$ 0.14	40.5 $\pm$ 4.2***
2	2.01 $\pm$ 1.4	1.71 $\pm$ 0.15	1.88 $\pm$ 11

The mixture of two peptidase inhibitors, amastatin and captopril, was added 10 min before the enkephalin administration at the final concentration of 1 μ M each. Phosphoramidon was given immediately after the mixture administration. Each value represents the mean $\pm$ S.E. of 4 experiments. ***P<0.01.

Table 4. Enhancing effect of captopril on the inhibitory potency of [Met⁵]-enkephalin in the rat vas deferens pretreated with both amastatin and phosphoramidon

Captopril (μ M)	IC ₅₀ (μ M)	Ratio of potency	% Difference
0.1	1.28 $\pm$ 0.15	1	24.7 $\pm$ 0.26***
1	0.96 $\pm$ 0.11	1.32 $\pm$ 0.042	0.911 $\pm$ 5.7
2	0.98 $\pm$ 0.10	1.30 $\pm$ 0.092	

The mixture of two peptidase inhibitors, amastatin and phosphoramidon, was added 10 min before the enkephalin administration at the final concentration of 1 μ M each. Captopril was given immediately after the mixture administration. Each value represents the mean $\pm$ S.E. of 4 experiments. ***P<0.01.

Table 5. Negative enhancing effect of L-tyrosyl-L-tyrosine (Tyr-Tyr) or D-phenylalanine (D-Phe) on the inhibitory potency of enkephalin in the rat vas deferens pretreated with three peptidase inhibitors, amastatin (Ama), phosphoramidon (Pho) and captopril (Cap)

Inhibitors	N ^a	IC ₅₀ (μ M)	Ratio of potency	% Difference
Ama+Pho+Cap	7	1.10 $\pm$ 0.14	1	0.571 $\pm$ 7.3
Ama+Pho+Cap+Tyr-Tyr	7	1.07 $\pm$ 0.10	1.03 $\pm$ 0.081	
Ama+Pho+Cap	4	1.06 $\pm$ 0.14	1	1.28 $\pm$ 1.5
Ama+Pho+Cap+D-Phe	4	1.05 $\pm$ 0.13	1.00 $\pm$ 0.016	

The final concentration of Ama, Pho or Cap was 1 μ M while that of Tyr-Tyr or D-Phe was 0.1 mM. The peptidase inhibitor was given 10 min before the [Met⁵]-enkephalin administration. ^aNumber of experiments.

examined. The IC₅₀ value of enkephalin in the presence of 1 μ M of captopril was not significantly different from that of 2 μ M of captopril, but significantly lower than that of 0.1 μ M of captopril (Table 4). This suggested that the enkephalin-hydrolyzing peptidyl dipeptidase A in rat vas deferens was inhibited maximally with captopril at the final concentration of 1 μ M, being consistent with the previous results obtained with guinea-pig ileum (1), mouse vas deferens (2) and a membrane preparation from the electric organ of *Torpedo marmorata* (14).

Finally, the effect of either L-tyrosyl-L-tyrosine, an inhibitor of an aminoterminal-directed dipeptidylpeptidase (15), or D-phenylalanine, a carboxypeptidase inhibitor (16), on the inhibitory potency of [Met⁵]-enkephalin in the rat vas deferens pretreated with three peptidase inhibitors, amastatin, phosphoramidon and captopril, at the final concentration of 1 μ M each was estimated. The IC₅₀ value of enkephalin was not significantly altered by the addition of either L-

tyrosyl-L-tyrosine or D-phenylalanine at the final concentration of 0.1 mM each (Table 5).

Discussion

Three enzymes, amastatin-sensitive aminopeptidase, phosphoramidon-sensitive endopeptidase-24.11 and captopril-sensitive peptidyl dipeptidase A, are indicated to be involved in the inactivation of [Met⁵]-enkephalin in rat vas deferens in the present investigation, since any of three peptidase inhibitors, amastatin, phosphoramidon and captopril, is shown to enhance significantly the inhibitory potency of [Met⁵]-enkephalin in the rat vas deferens pretreated with the residual two peptidase inhibitors. Additionally, the enkephalin-hydrolyzing aminopeptidase, endopeptidase-24.11 and peptidyl dipeptidase A in rat vas deferens are suggested to be inhibited maximally with 1 μ M of amastatin, 1 μ M of phosphoramidon and 1 μ M of captopril, respectively, since the enhancing effect of each peptidase inhibitor on the enkephalin-induced inhibition is shown to

reach maximum at the dose of 1 μ M. In contrast to these three peptidases, both dipeptidyl aminopeptidase and carboxypeptidase seem not to be involved significantly in the inactivation of exogenously added enkephalin in rat vas deferens since the IC₅₀ values of [Met⁵]-enkephalin in the preparations pretreated with three peptidase inhibitors, amastatin, phosphoramidon and captopril, are not significantly different from those pretreated with four peptidase inhibitors, amastatin, phosphoramidon, captopril and either L-tyrosyl-L-tyrosine, which has been demonstrated to be an inhibitor of an amino-terminal-directed dipeptidylpeptidase (15), or D-phenylalanine, which has been shown to be a carboxypeptidase inhibitor (16).

The results obtained with rat vas deferens in the present investigation coupled with those obtained with both guinea-pig ileum (1) and mouse vas deferens (2) in the previous studies show that three distinct enzymes, amastatin-sensitive aminopeptidase, phosphoramidon-sensitive endopeptidase-24.11 and captopril-sensitive peptidyl dipeptidase A, play critical roles in the inactivation of enkephalin in three isolated preparations, guinea-pig ileum, mouse vas deferens and rat vas deferens. Additionally, the data obtained with these three isolated preparations indicate that both L-tyrosyl-L-tyrosine-sensitive dipeptidyl aminopeptidase and D-phenylalanine-sensitive carboxypeptidase are not involved significantly in the inactivation of enkephalin in three isolated preparations, guinea-pig ileum (1), mouse vas deferens (2) and rat vas deferens. Moreover, the finding that [Met⁵]-enkephalin remains intact almost wholly after it is incubated with either ileal or striatal membrane fractions of guinea-pig in the presence of three peptidase inhibitors, amastatin, thiorphan and captopril, at the final concentration of 1 μ M each, for 60 min at 37°C (3), strongly indicates that enkephalin is exclusively inactivated by three enzymes.

However, the total activity of three enkephalin-inactivating enzymes in one isolated preparation is likely to be significantly different from that in the other, since the

magnitude of the enhancement of the inhibitory potency of [Met⁵]-enkephalin by the mixture of three peptidase inhibitors, amastatin, phosphoramidon and captopril, at the final concentration of 1 μ M each, in mouse vas deferens has been shown to be approximately three-fold higher than that in guinea-pig ileum, but two- to three-fold lower than that in rat vas deferens (7). Additionally, the mixture of these three peptidase inhibitors has been shown not to enhance significantly the inhibitory potency of β -endorphin in both guinea-pig ileum and rat vas deferens but augment significantly the potency of β -endorphin in mouse vas deferens (7).

In conclusion, the rat vas deferens, like the other three preparations, guinea-pig ileum (1), mouse vas deferens (2) and striatal membranes of guinea-pig brain (3), is shown to contain three distinct enkephalin-inactivating enzymes. Additionally, the susceptibility of the enkephalin-hydrolyzing enzymes to inhibitors in rat vas deferens is found to be similar to that in both guinea-pig ileum (1) and mouse vas deferens (2), although the peptidases in rat vas deferens have been shown to be different from those in mouse vas deferens in terms of the ability of the β -endorphin hydrolysis (7).

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