Inactivation of Dynorphin-(1-8) in Isolated Preparations by Three Peptidases

Hiroaki NUMATA, Toyokazu HIRANUMA and Tetsuo OKA*

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

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Abstract—Inactivation of dynorphin-(1-8) in three in vitro isolated preparations, guinea-pig ileum, mouse vas deferens and rabbit vas deferens, was estimated by employing the relatively specific inhibitors of enkephalin-hydrolyzing enzymes. All three enzyme inhibitors, amastatin, captopril and phosphoramidon, significantly enhanced the inhibitory potency of dynorphin-(1-8) in the three isolated preparations. The magnitude of the enhancement of the dynorphin potency by captopril was significantly higher than that by either amastatin or phosphoramidon in guinea-pig ileum; that by amastatin was significantly higher than that by either captopril or phosphoramidon in rabbit vas deferens; and that by amastatin was similar to that by captopril, but significantly higher than that by phosphoramidon in mouse vas deferens. The K_i values of three antagonists, naloxone, Mr 2266 and ICI 154129, against dynorphin-(1-8) in the presence of the three peptidase inhibitors indicated that dynorphin-(1-8) acted on kappa receptors in guinea-pig ileum and on both kappa and delta receptors in mouse vas deferens. Since amastatin, captopril and phosphoramidon produced the naloxone-reversible inhibition of contractions of guinea-pig ileum in the presence of dynorphin-(1-8), all three dynorphin-inactivating enzymes were indicated to be located very close to kappa receptors.

Enkephalins have been shown to be inactivated by three enzymes, amastatin-sensitive aminopeptidase, captopril-sensitive peptidyl dipeptidase A (angiotensin I converting enzyme, kininase II, peptidyl dipeptide hydrolase; EC 3.4.15.1) and phosphoramidon-sensitive endopeptidase-24.11 ("enkephalinase"; EC 3.4.24.11), in three in vitro isolated preparations, guinea-pig ileum (1), mouse vas deferens (2) and rat vas deferens (3). Since these enkephalin-hydrolyzing enzymes are located very close to opioid receptors (1-3), these enzymes are suggested not only to inactivate the exogenously given enkephalin in the vicinity of opioid receptors but also suggested to play important roles in the termination of the action on opioid receptors of the enkephalin released from presynaptic nerve terminals.

Additionally, enkephalin has been demonstrated to be almost exclusively hydrolyzed by these three enzymes, since enkephalin remains intact almost totally after it is incubated with ileal or striatal membrane preparations of guinea-pig for 60 min at 37°C in the presence of the inhibitors against these three enzymes, although enkephalin is completely hydrolyzed and free tyrosine and the Tyr-Gly-Gly fragment are produced when the incubation mixture does not contain the peptidase inhibitor (4).

Dynorphin-(1-8), another endogenous opioid peptide, has been suggested to be also inactivated by peptidases in isolated preparations such as rabbit vas deferens since its inhibitory action on the electrically-evoked contractions of the isolated preparation has been shown to be significantly enhanced by the pretreatment of the preparation with the mixture of peptidase inhibitors (5, 6). The

* To whom reprint requests should be addressed.

enzyme involved in the inactivation of dynorphin-(1-8), however, is unknown at present. In the present investigation, therefore, it was examined whether or not dynorphin-(1-8) was inactivated by three enkephalin-hydrolyzing enzymes in three isolated preparations guinea-pig ileum, mouse vas deferens and rabbit vas deferens.

Additionally, the preference of the opioid-receptor type by dynorphin-(1-8) was re-examined in isolated preparations in the presence of peptidase inhibitors by employing the three opioid antagonists, since dynorphin-(1-8) had been reported to act on either kappa receptors in the preliminary experiments (7) or a different opioid-receptor type than the three classical opioid-receptor types, mu, kappa and delta type (8), and dynorphin-(1-9) had been indicated to act on both kappa and delta receptors (9).

Moreover, it was estimated in the present investigation whether or not dynorphin-inactivating enzymes were located very close to kappa receptors in guinea-pig ileum, since enkephalin-inactivating enzymes had been indicated to be located very close to mu receptors in guinea-pig ileum in the previous investigation (1).

Materials and Methods

Chemicals: Gifts of compounds which were gratefully received were dynorphin-(1-8) from Prof. Y. Kiso, Kyoto Pharmaceutical University (Kyoto); ICI 154129 (N,N-diallyl-Tyr-Gly- ψ -(CH₂S)-Phe-Leu) (where ψ -(CH₂S) signifies replacement of the amide CO-NH bond by CH₂S) from Dr. J.W. Holaday, Walter Reed Army Inst. Res. (Washington, D.C., U.S.A.); Mr 2266 [(-)-2-(3-furylmethyl)-5,9-diethyl-2'-hydroxy-6,7-benzomorphan] from Nippon CH Boehringer Sohn Co., Ltd. (Osaka); and captopril and naloxone from Sankyo Company (Tokyo). Amastatin and phosphoramidon were purchased from Peptide Institute, Inc. (Minoh).

In vitro isolated preparations: Male ICR-JCL mice weighing 30–50 g, male Hartley guinea-pigs weighing 300–600 g, and male Japanese White rabbits weighing 2.5–3.5 kg were used for this study. The vasa deferentia from mice or rabbits, and the myenteric plexus-longitudinal muscle strip of guinea-

pig ileum were prepared and set up for electrical stimulation as described previously (10, 11). The % inhibition of electrically-evoked muscle contractions produced by an opioid peptide was plotted against the log concentration of the peptide to estimate the IC₅₀ (concentration of the opioid peptide to produce 50% inhibition of contractions). Amastatin, captopril or phosphoramidon at the final concentration of 1 μ M each was employed as an inhibitor of each peptidase in the present investigation since the previous studies had shown that the enkephalin-hydrolyzing aminopeptidase, peptidyl dipeptidase A and endopeptidase-24.11 were inhibited almost completely with 1 μ M of amastatin, 1 μ M of captopril, and 1 μ M of phosphoramidon, respectively (1–4). Either a peptidase inhibitor or the mixture of two or three peptidase inhibitors was given 10 min before the opioid administration. The ratio of the potency and the % difference, shown in the tables, were calculated from the following formulas: ratio of potency = IC₅₀ before additional treatment/IC₅₀ after additional treatment and % difference = [(IC₅₀ before additional treatment – IC₅₀ after additional treatment)/IC₅₀ before additional treatment] $\times$ 100. The statistical significance of % differences between IC₅₀ values of two adjacent groups shown in the tables was determined by the paired Student's *t*-test. The K_e (equilibrium dissociation constant) values of opioid antagonists against dynorphin-(1-8) were determined by the 'single' dose method (12).

Results

Enhancing effects of peptidase inhibitors on the potency of dynorphin-(1-8): Dynorphin-(1-8) dose-dependently inhibited the electrically-evoked contractions of all isolated preparations employed in the present investigation. Its IC₅₀ value in guinea-pig ileum, mouse vas deferens and rabbit vas deferens was significantly decreased by the pretreatment of the preparation with mixture of three peptidase inhibitors, amastatin, captopril and phosphoramidon, at the final concentration of 1 μ M each at 10 min before the dynorphin administration (Table 1). Each peptidase inhibitor was then administered individually in the following experiments in

order to determine which peptidase inhibitor had an enhancing effect on the inhibitory potency of dynorphin-(1-8).

In three isolated preparations, especially in guinea-pig ileum, the IC₅₀ value of dynorphin-(1-8) in one preparation was sometimes markedly different from that in another in the absence of the peptidase inhibitor. Thus, the mean of the IC₅₀ values of dynorphin-(1-8) in one control group was sometimes prominently different from that in the other control group (Tables 2, 3 and 4). When

the effect of a particular peptidase inhibitor on the inhibitory potency of dynorphin was examined, therefore, the IC₅₀ values of dynorphin before and after the addition of a particular peptidase inhibitor were estimated in the same preparation.

All three peptidase inhibitors significantly enhanced the inhibitory potency of dynorphin-(1-8) in either guinea-pig ileum (Table 2), mouse vas deferens (Table 3) or rabbit vas deferens (Table 4).

In guinea-pig ileum, the magnitude of the

Table 1. Enhancing effects of peptidase inhibitors on the inhibitory potency of dynorphin-(1-8) in isolated preparations

Preparations	N ^a	IC ₅₀ (nM)		Ratio of potency ^c
		Without inhibitors	With inhibitors ^b	
Guinea-pig ileum	5	320 ± 160	10.6 ± 4.7	43.1 ± 15
Mouse vas deferens	4	91.8 ± 9.6	7.93 ± 0.45	11.6 ± 1.0
Rabbit vas deferens	4	2090 ± 1400	104 ± 80	26.4 ± 3.9

Each value represents the mean ± S.E. ^aNumber of experiments. ^bThe mixture of three peptidase inhibitors, amastatin, captopril and phosphoramidon, at the final concentration of 1 μM each was given 10 min before the dynorphin administration. ^cIC₅₀ without inhibitors/IC₅₀ with inhibitors.

Table 2. The enhancing effects of peptidase inhibitors on the inhibitory potency of dynorphin-(1-8) in guinea-pig ileum

Inhibitors	N ^a	IC ₅₀ (nM)	Ratio of potency	% Difference
None	7	884 ± 54	1	45.1 ± 3.4***
Amastatin	7	464 ± 27	1.86 ± 0.12	
None	8	323 ± 10	1	79.8 ± 2.6***
Captopril	8	58.0 ± 16	5.58 ± 0.75	
None	6	98.7 ± 28	1	22.9 ± 5.0***
Phosphoramidon	6	73.0 ± 17	1.33 ± 0.086	

Each value represents the mean ± S.E. ^aNumber of experiments. ***P < 0.01.

Table 3. The enhancing effects of peptidase inhibitors on the inhibitory potency of dynorphin-(1-8) in mouse vas deferens

Inhibitors	IC ₅₀ (nM)	Ratio of potency	% Difference
None	69.6 ± 19	1	54.8 ± 5.4***
Amastatin	26.7 ± 3.7	2.45 ± 0.30	
None	50.5 ± 8.0	1	54.0 ± 6.7***
Captopril	20.7 ± 3.4	2.50 ± 0.33	
None	72.7 ± 18	1	28.2 ± 3.4***
Phosphoramidon	54.9 ± 15	1.42 ± 0.071	

Each value represents the mean ± S.E. of 8 experiments. ***P < 0.01.

Table 4. The enhancing effects of peptidase inhibitors on the inhibitory potency of dynorphin-(1-8) in rabbit vas deferens

Inhibitors	IC ₅₀ (μ M)	Ratio of potency	% Difference
None	0.578 $\pm$ 0.18	1	82.4 $\pm$ 2.3***
Amastatin	0.107 $\pm$ 0.039	6.30 $\pm$ 1.2	
None	1.07 $\pm$ 0.51	1	50.5 $\pm$ 6.1***
Captopril	0.491 $\pm$ 0.26	2.23 $\pm$ 0.42	
None	1.34 $\pm$ 0.67	1	30.7 $\pm$ 3.7***
Phosphoramidon	0.949 $\pm$ 0.46	1.46 $\pm$ 0.081	

Each value represents the mean $\pm$ S.E. of 5 experiments. ***P<0.01.

enhancement of the dynorphin potency by amastatin was significantly higher than that by phosphoramidon, but significantly lower than that by captopril (Table 2).

The degree of the augmentation of the dynorphin potency by amastatin was similar to that by captopril, but significantly higher than that by phosphoramidon in mouse vas deferens (Table 3).

In rabbit vas deferens, the magnitude of the enhancement of the dynorphin potency by amastatin was significantly higher than that by captopril, and that by captopril was significantly higher than that by phosphoramidon (Table 4).

The inhibitory action of the peptidase inhibitor on contractions of guinea-pig ileum in the presence of dynorphin-(1-8): Either amastatin, captopril or phosphoramidon inhibited the electrically-evoked contractions of guinea-pig ileum in the presence of dynorphin-(1-8) (Fig. 1), although these peptidase inhibitors by themselves did not depress the contractions in the absence of an opioid peptide (figures were not shown). The magnitude of the inhibition by each peptidase inhibitor, although it was slightly altered by changing the administration order of the peptidase inhibitor, was the highest by captopril, the lowest by phosphoramidon and intermediate by amastatin (Fig. 1). The inhibition induced by peptidase inhibitors in the presence of dynorphin-(1-8) was antagonized by naloxone (Fig. 1). The degree of the antagonism by naloxone was not complete in some preparations as shown in Fig. 1, but complete in other preparations. The apparent incomplete antagonism by naloxone was likely to be produced by the agonistic action

Fig. 1. Naloxone-reversible inhibitory actions of peptidase inhibitors on electrically-evoked contractions of guinea-pig ileum in the presence of dynorphin-(1-8). Compounds were given to the bath at the dot. DYN, 5 nM of dynorphin-(1-8). A, 1 μ M of amastatin. C, 1 μ M of captopril. P, 1 μ M of phosphoramidon. Nal, 1 μ M of naloxone.

of relatively high doses of naloxone on some preparations since dynorphin-(1-8) at doses below 5×10^{-9} M in the presence of the three peptidase inhibitors did not inhibit the contractions at all in all preparations after the administration of relatively high doses (e.g., 10^{-6} M) of naloxone which produced the slight inhibition of contractions by itself in some preparations.

The K_e values of opioid antagonists against dynorphin-(1-8) in the presence of peptidase: The K_e values against dynorphin-(1-8) of naloxone, which had been shown to have a high affinity to mu receptors (13), were relatively high, while those of Mr 2266, which had been reported to have a high affinity to both mu and kappa receptors (13), were relatively low in guinea-pig ileum (Table 5). Additionally, the K_e values of ICI 154129, which had been found to have a relatively high affinity to delta receptors, a low affinity to mu receptors, and a very low affinity to kappa receptors (13), were very high in 3 among 6 preparations and they were not able

Table 5. The K_e values of naloxone, Mr 2266 and ICI 154129 against dynorphin-(1-8) in isolated preparations

Preparations	K_e (nM)		
	Naloxone	Mr 2266	ICI 154129
Guinea-pig ileum	16.6±3.6	4.42±1.1	— ^a
Mouse vas deferens	41.3±7.9	3.67±0.76	614±110

The K_e values were estimated in the presence of the mixture of three peptidase inhibitors, amastatin, captopril and phosphoramidon, at the final concentration of 1 μ M each. Each value represents the mean±S.E. of 4 experiments. ^aAntagonism by ICI 154129 at the dose of 10 μ M was not observed in 3 among 6 preparations, and K_e values in the residual 3 preparations were 9.43, 12.7 and 23.3 μ M.

to be estimated in the remaining 3 preparations of guinea-pig ileum because of no antagonism by ICI 154129 (Table 5).

In mouse vas deferens, the K_e values against dynorphin-(1-8) of naloxone, Mr 2266 and ICI 154129 were relatively high, relatively low and relatively low, respectively (Table 5).

Discussion

The present investigation indicates that dynorphin-(1-8) can be inactivated by three enzymes, amastatin-sensitive aminopeptidase, captopril-sensitive peptidyl dipeptidase A and phosphoramidon-sensitive endopeptidase-24.11, in three in vitro isolated preparations, guinea-pig ileum, mouse vas deferens and rabbit vas deferens, since the inhibitory potency of dynorphin-(1-8) has been shown to be significantly enhanced by the pretreatment of the preparation with either amastatin, captopril or phosphoramidon in all three preparations.

The fact that the magnitude of the enhancement of the dynorphin potency by captopril was significantly higher than that by either amastatin or phosphoramidon in guinea-pig ileum suggests that captopril-sensitive peptidyl dipeptidase A plays the most important role in the inactivation of dynorphin-(1-8) in guinea-pig ileum. However, the important role of amastatin-sensitive aminopeptidase as well as captopril-sensitive enzyme in the inactivation of dynorphin-(1-8) in mouse vas deferens is suggested by the finding that the degree of the enhancement of the dynorphin potency by amastatin was similar to that by captopril but significantly higher than that

by phosphoramidon in mouse vas deferens. In rabbit vas deferens, amastatin-sensitive aminopeptidase is likely to play the most important role in the inactivation of dynorphin-(1-8) since the magnitude of the augmentation of the dynorphin potency by amastatin has been shown to be significantly higher than that by either captopril or phosphoramidon. The minor role of phosphoramidon-sensitive endopeptidase-24.11 in the inactivation of dynorphin-(1-8) in all three preparations is indicated by the observation that the magnitude of the enhancement of the dynorphin potency by phosphoramidon was lower than that by either amastatin or captopril in the three preparations.

The data in the present investigation along with those obtained previously (14, 15) show that the relative importance of the three enzymes in the inactivation of dynorphin-(1-8) in guinea-pig ileum is different from that of either [Met⁵]- or [Leu⁵]-enkephalin since the magnitude of the enhancement of the inhibitory potency of both [Met⁵]- and [Leu⁵]-enkephalin by amastatin has been shown to be significantly higher than that by either captopril or phosphoramidon in guinea-pig ileum (14, 15). Additionally, the relative importance of the three enzymes in the inactivation of dynorphin-(1-8) in mouse vas deferens is different from that of [Met⁵]-enkephalin but similar to that of [Leu⁵]-enkephalin since the magnitude of the augmentation of the [Met⁵]-enkephalin potency by amastatin is similar to that by either captopril or phosphoramidon in mouse vas deferens (14), while the magnitude of the enhancement of [Leu⁵]-enkephalin potency by

amastatin is similar to that by captopril but higher than that by phosphoramidon in mouse vas deferens (15).

The reports that naloxone has a high affinity to mu receptors (K_e values ranging from 1 to 5 nM) and relatively low affinity to both kappa and delta receptors (K_e values ranging from 10 to 50 nM) (10, 13, 16, 17); Mr 2266 has a high affinity to both mu and kappa receptors (K_e values ranging from 1 to 5 nM) and relatively low affinity to delta receptors (K_e values ranging from 10 to 50 nM) (10, 13, 17); and ICI 154129 has a relatively high affinity to delta receptors (K_e values ranging from 0.1 to 0.9 μ M), the lowest affinity to kappa receptors (K_e values being more than 20 μ M) and intermediate affinity to mu receptors (K_e values ranging from 7 to 8 μ M) (13, 18, 19) indicate that dynorphin-(1-8) acts on kappa receptors in guinea-pig ileum and on both kappa and delta receptors in mouse vas deferens.

The fact that the relative importance of the three enzymes in the inactivation of dynorphin-(1-8) is different from that of enkephalins may be caused by the fact that one opioid peptide is hydrolyzed more easily than the other by a particular enzyme as shown by the previous reports (14), although the probability that the relative activity of three enzymes in the vicinity of mu receptors is different from that of kappa receptors can not be neglected since both [Met⁵]- and [Leu⁵]-enkephalin have been shown to act on mu receptors in guinea-pig ileum and on delta receptors in mouse vas deferens (13, 16), while dynorphin-(1-8) has been suggested to act on kappa receptors in guinea-pig ileum and on both kappa and delta receptors in mouse vas deferens in the present investigation.

The preliminary experiment by employing crude enzyme preparations obtained from membrane fractions of guinea-pig ileum and striatum has shown that dynorphin-(1-8) is hydrolyzed by amastatin-sensitive aminopeptidase at the Tyr-Gly bond, by phosphoramidon-sensitive endopeptidase-24.11 at the Gly-Phe bond, and captopril-sensitive peptidyl dipeptidase A at both Arg-Arg and Phe-Leu bonds (Hiranuma, T. and Oka, T., unpublished observation). Since [Leu⁵]-

enkephalin-Arg⁶, an initial hydrolysis product of dynorphin-(1-8) by the captopril-sensitive enzymes, still has essentially the same potency as dynorphin-(1-8) in mouse vas deferens (5), the observation that captopril has significantly enhanced the potency of dynorphin-(1-8) in mouse vas deferens indicates that [Leu⁵]-enkephalin-Arg⁶ is further hydrolyzed by captopril-sensitive peptidase to Tyr-Gly-Gly-Phe which is reported to have, if any, very weak potency on opioid receptors (20).

All three enkephalin-hydrolyzing enzymes have been suggested to be very closely located to mu receptors in the guinea-pig ileum (1) and to delta receptors in mouse vas deferens (2) by employing enkephalin which has been indicated to act on mu receptors in guinea-pig ileum (13, 16) and delta receptors in mouse vas deferens (13, 16). In the present investigation, these three enzymes were indicated to very closely located to kappa receptors in guinea-pig ileum by employing dynorphin-(1-8), which has been suggested to act on kappa receptors in guinea-pig ileum in the present study, since these enzymes have been shown to produce a significant concentration difference of dynorphin-(1-8) between the surrounding organ bath and the vicinity of kappa receptors as demonstrated in Fig. 1, and such concentration difference can be produced only when these inactivating-enzymes are located very closely to kappa receptors. The previous (1-3) and present investigation, therefore, strongly suggest that there are three opioid-peptide-inactivating enzymes in the vicinity of three types of opioid receptors, mu, kappa and delta receptors, as well as an unidentified type of opioid receptor in rat vas deferens.

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