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[Met^5]enkephalin-Arg-Gly-Leu-induced antinociception is greatly increased by peptidase inhibitors

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Abstract

Previous in vitro studies showed that the degradation of [Met^5]enkephalin-Arg-Gly-Leu by cerebral membrane preparations is almost completely prevented by a mixture of three peptidase inhibitors: amastatin, captopril and phosphoramidon. The present investigations showed that the inhibitory effect of [Met^5]enkephalin-Arg-Gly-Leu administered intra-third-ventricularly on the tail-flick response was increased more than 1000-fold by the intra-third-ventricular pretreatment of rats with three peptidase inhibitors. The inhibition produced by the enkephalin octapeptide in rats pretreated with any combination of two peptidase inhibitors was significantly smaller than that in rats pretreated with three peptidase inhibitors, indicating that any residual single peptidase could inactivate significant amounts of the octapeptide. The present data, together with those obtained from previous studies, clearly show that three types of enzymes, amastatin-, captopril- and phosphoramidon-sensitive enzymes, play important roles in the inactivation of endogenous opioid penta- and octa-peptides administered intra-third-ventricularly to rats.

Keywords: [Met^5]enkephalin-Arg-Gly-Leu; Opioid peptide; Antinociception; Peptidase inhibitor

1. Introduction

It has been shown that when [Met^5]enkephalin-Arg-Gly-Leu is incubated with ileal and striatal membrane fractions for 60 min at 37 °C in the presence of three peptidase inhibitors, amastatin, captopril and phosphoramidon, approximately 95% of the enkephalin octapeptide remains intact, while in the absence of the peptidase inhibitors, the octapeptide is completely hydrolyzed during the initial 15-min incubation (Hiranuma et al., 1997). This shows that [Met^5]enkephalin-Arg-Gly-Leu is hydrolyzed, at least in these membrane preparations, by only three types of membrane-bound enzymes: amastatin-sensitive aminopeptidase(s), captopril-sensitive dipeptidyl carboxypeptidase I (angiotensin I-converting enzyme, kininase II, EC 3.4.15.1) and phosphoramidon-sensitive endopeptidase-24.11 ("enkephalinase", EC 3.4.24.11). Additionally, the close proximity of these enzymes to the opioid receptors in isolated preparations such as guinea pig ileum (Aoki et al., 1984), mouse vas deferens (Aoki et al., 1986) and rat vas deferens (Cui et al., 1986), suggests that they act to terminate the physiological action of [Met^5]enkephalin-Arg-Gly-Leu.

Because the products of [Met^5]enkephalin-Arg-Gly-Leu hydrolysis by either amastatin- or phosphoramidon-sensitive enzymes such as free Tyr and the Tyr-Gly-Gly, [des-Tyr]- and [des-Tyr-Gly-Gly][Met^5]enkephalin-Arg-Gly-Leu fragments are suggested to have very low, if any, agonist activity at opioid receptors (Morley, 1980), the potency of the octapeptide should be decreased by its hydrolysis with these two peptidases. In fact, the potency of [Met^5]enkephalin-Arg-Gly-Leu has been shown to be significantly increased by either amastatin or phosphoramidon (Hiranuma et al., 1997). In contrast, [Met^5]enkephalin-Arg, the initial hydrolysis product of [Met^5]enkephalin-Arg-Gly-Leu by captopril-sensitive enzyme, has been shown to be approximately threefolds more potent than [Met^5]enkephalin-Arg-Gly-Leu at μ-opioid receptors in guinea pig ileum, although Tyr-Gly-Gly-Phe, the second hydrolysis product, is approximately 30-folds less potent than [Met^5]enkephalin-Arg-

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Gly-Leu (Hiranuma et al., 1997). The change in the potency of [Met^5]enkephalin-Arg-Gly-Leu after its hydrolysis by the captopril-sensitive enzyme, therefore, depends on the balance between the rate of the metabolic change from [Met^5]-enkephalin-Arg-Gly-Leu to [Met^5]enkephalin-Arg and that from [Met^5]enkephalin-Arg to the Tyr-Gly-Gly-Phe fragment. Thus, it was of interest to examine the in vivo effects of [Met^5]enkephalin-Arg-Gly-Leu on the central nervous system in the presence or the absence of the peptidase inhibitors. In the present investigation, effects of the three peptidase inhibitors were examined on the antinociception induced by the intra-third-ventricular administration of [Met^5]enkephalin-Arg-Gly-Leu.

2. Materials and methods

2.1. Chemicals

Captopril and naloxone HCl were kindly provided by Sankyo (Tokyo, Japan). Other chemicals were purchased from the following sources: [Met^5]enkephalin, amastatin and phosphoramidon from Peptide Institute, Minoh, Japan; and [Met^5]enkephalin-Arg-Gly-Leu from Peninsula Laboratories, Belmont, CA, USA. All chemicals were dissolved in saline. The stock solution for all peptides used was prepared at concentrations of 0.1–10 mM in siliconized plastic tubes, kept at − 18 °C and then diluted to the desired concentration just before use.

2.2. Intra-third-ventricular microinjection

Male Wistar rats weighing 180–220 g were anesthetized with pentobarbital sodium (40 mg/kg, i.p.), were mounted on a stereotaxic frame and implanted with a stainless-steel injection cannula (external diameter, 0.30 mm) 5–7 days prior to the day of the experiment. The lower end of the injection cannula was aimed at the third cerebral ventricle (6.0 mm anterior from lambda and 7.8 mm ventral from the surface of the skull) according to the atlas of Paxinos and Watson (1986). The injection cannula was attached to a motor-driven 50-μl microsyringe by polyethylene tubing. Drugs were injected in volumes of 10 μl for 1 min, and the connecting tube was removed from the injection cannula 1 min after the injection. The distribution of the drug solution in the cerebroventricular system was verified by infusion of methylene blue dissolved in saline after the experiment.

2.3. Tail-flick response

The antinociceptive effect of opioids was measured by the tail immersion assay with 55 °C as the nociceptive stimulus (Janssen et al., 1963). The latency to flick the tail from the 55 °C water was measured before and 5, 10, 15, 30, 45, 60, 75, 90, 105 and 120 min after the opioid injection. The latency to flick the tail before the injection was approximately 1 s (0.5–1.7 s). A cut-off time of 5 s was used to prevent any injury to the tail. The percent of the maximal possible effect (MPE) for each animal at each time was calculated using the following formula: %MPE=[(test latency − baseline latency)/(5 − baseline latency)] × 100. The area under the curve (AUC) value for the antinociceptive action of an opioid on each rat was calculated for some experiments.

2.4. Doses of each peptidase inhibitor to inhibit the targeted peptidase in the in vivo experiments

Our previous study had shown that the maximum inhibition of the tail-flick response by intra-third-ventricular administration of [Met^5]enkephalin was achieved in rats pretreated with the mixture of three peptidase inhibitors, amastatin, captopril and phosphoramidon, at the doses of 10 nmol each in dose-increasing experiments with the peptidase inhibitors (Taniguchi et al., 1998). Furthermore, previous in vitro experiments had shown that the concentration of three peptidase inhibitors required to inhibit the hydrolysis of [Met^5]enkephalin was essentially the same as that of [Met^5]enkephalin-Arg-Gly-Leu (Hiranuma and Oka, 1986; Hiranuma et al., 1997). Therefore, 10 nmol was selected as the dose of each peptidase inhibitor to inhibit the targeted peptidase in the present study.

2.5. In vivo apparent pA2 analysis

The tail-flick latency of rats pretreated with the three peptidase inhibitors was measured before and 15 min after the intra-third-ventricular injection of [Met^5]enkephalin-Arg-Gly-Leu and converted to %MPE. The dose–effect curve for the agonist in each rat was obtained by injecting a rat with two or three doses, such as 0.5, 1 and 2 nmol with a 48-h interinjection interval. Individual ED_{50} values were calculated by least-squares regression, using the portion of the dose–effect curve spanning the 50% MPE. The mean ED_{50} value was obtained from individual ED_{50} values. Naloxone was given subcutaneously 5 min before the intra-third-ventricular administration of the agonist. Dose ratio was calculated by dividing each ED_{50} value in the presence of naloxone by mean ED_{50} value in the absence of naloxone. The pooled pA_2 value was determined by entering all the dose ratio values (Tallarida and Murray, 1987).

2.6. In vitro isolated preparations

Male ICR-Jcl mice weighing 40–50 g and male Hartley guinea pig weighing 400–600 g were used for this study. The mouse vas deferens and the myenteric plexus longitudinal muscle strip of guinea pig ileum were set up for electrical stimulation as described previously (Oka et al., 1982). The percent inhibition of the stimulated muscle twitch produced by an opioid was plotted against the log concentration of the opioid to estimate the IC_{50} (opioid

concentration producing 50% inhibition of the twitch). When the effect of peptidase inhibitors on the IC_{50} value of an opioid peptide was studied, these were given 5 min before the administration of the opioid peptide. The ratio of the potency and the percent difference, shown in Table 1, were calculated from the following formulas: ratio of potency = IC_{50} without peptidase inhibitor/IC_{50} with peptidase inhibitors, and %difference=[(IC_{50} without peptidase inhibitor − IC_{50} with peptidase inhibitors)/IC_{50} without peptidase inhibitor] × 100.

2.7. Statistical analyses

The data were analyzed by one-way analysis of variance (ANOVA) followed by Scheffe's *F*-test as described in the legend for Fig. 4. The statistical significance of percent differences between IC_{50} values of two adjacent groups shown in Table 1 was determined by the paired Student's *t*-test. SPSS 11.0J (SPSS Japan, Tokyo, Japan) for Windows (Microsoft, Redmond, WA, USA) was used for all analyses.

3. Results

3.1. Effects of the mixture of two or three peptidase inhibitors on [Met^5]enkephalin-Arg-Gly-Leu-induced inhibition of tail-flick response

Previous in vitro studies (Norman and Chang, 1985; Hiranuma et al., 1997) indicated that the captopril-sensitive peptidase plays the most important role among the three peptidases in the hydrolysis of [Met^5]enkephalin-Arg-Gly-Leu. However, the antinociceptive potency of the 10 nmol enkephalin octapeptide was not augmented at all by the intra-third-ventricular pretreatment of rats with 10 nmol captopril (data are not shown). Additionally, 10 nmol of amastatin alone or phosphoramidon alone also did not at all enhance the potency of the octapeptide (data are not shown). Therefore, the antinociceptive potency of the octapeptide was examined in rats pretreated with the mixture of the two or three peptidase inhibitors. The time course of changes in the inhibitory action on the tail-flick response after the intra-third-ventricular administration of 1 nmol [Met^5]enkephalin-Arg-Gly-Leu to rats pretreated intra-third-ventricularly with the two or three peptidase inhibitors at the doses of 10 nmol each is shown in Fig. 1. The antinociceptive action of [Met^5]enkephalin-Arg-Gly-Leu in rats pretreated with three peptidase inhibitors was significantly greater than that in rats pretreated with any combination of two peptidase inhibitors (Fig. 1).

3.2. Effects of naloxone on [Met^5]enkephalin-Arg-Gly-Leu-induced inhibition of tail-flick response

The dose–effect curves for [Met^5]enkephalin-Arg-Gly-Leu were shifted dose dependently to the right after pretreatment subcutaneously with 0.01, 0.02 and 0.05 mg/kg of naloxone HCl 5 min before the intra-third-ventricular administration of the agonist. Fig. 2 shows the Schild plots for naloxone with values derived from individual dose ratios for each rat. The pA_2 value for naloxone against [D-Ala^2, *N*-Me-Phe^4, Gly-ol]enkephalin (DAMGO), a selective μ-opioid receptor agonist, under the same experimental condition was reported previously (Kitamura et al., 2000), and the reported pooled pA_2 values and slopes (95% confidence limits shown in parentheses) are 7.50 (7.42–7.58) and −1.20 (1.40–1.00), respectively. The pooled pA_2 values and their 95% confidence limits shown in Fig. 2 demonstrate that the effectiveness of naloxone to antagonize the antinociceptive effects of [Met^5]enkephalin-Arg-Gly-Leu is similar to and not significantly different from that of DAMGO reported previously.

3.3. Inhibitory potency of [Met^5]enkephalin-Arg-Gly-Leu relative to that of [Met^5]enkephalin

The inhibition of the tail-flick response in rats pretreated with the three peptidase inhibitors by the intra-third-ventricular administration of [Met^5]enkephalin has been shown to be produced through μ-opioid receptors (Taniguchi et al., 1998). Therefore, the inhibitory potency of [Met^5]enkephalin-Arg-Gly-Leu was compared to that of [Met^5]enkephalin, another representative endogenous opioid peptide. The dose-dependent increase in the antinociceptive effects was

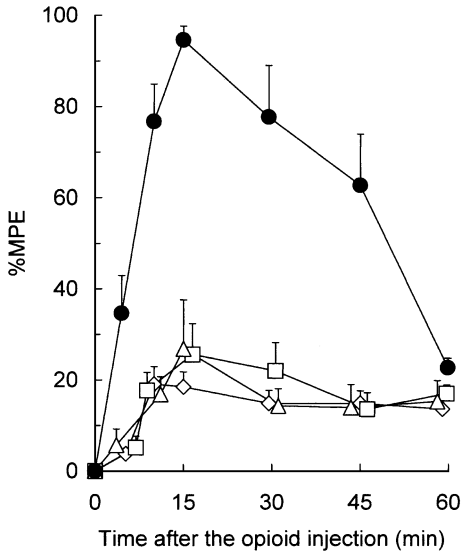

Fig. 1. Combined effects of two or three peptidase inhibitors, amastatin, captopril and phosphoramidon, on the [Met^5]enkephalin-Arg-Gly-Leu-induced inhibition of the tail-flick response in rats. The mixture of two or three peptidase inhibitors [◇, amastatin and captopril (n = 5); □, amastatin and phosphoramidon (n = 5); △, captopril and phosphoramidon (n = 5); or ●, amastatin, captopril and phosphoramidon (n = 9)] at the dose of 10 nmol each was given intra-third-ventricularly 10 min before the intra-third-ventricular administration of [Met^5]enkephalin-Arg-Gly-Leu (1 nmol). Each symbol represents the mean. Vertical bars represent the S.E.M. of n observations.

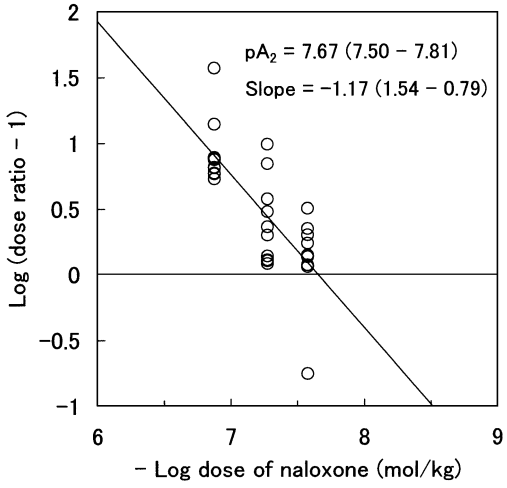

Fig. 2. Schild plots for naloxone in the antinociception assay of [Met^5]enkephalin-Arg-Gly-Leu. Abscissae: negative log unit of the molar doses of naloxone. Ordinates: log of (dose ratio −1). Each point was converted from individual dose ratios for each rat. The pooled pA_2 values and slopes are included in the panel. The 95% confidence limits are shown in parentheses.

observed on intra-third-ventricular administration of [Met^5]-enkephalin-Arg-Gly-Leu (0.01–1 nmol) or [Met^5]enkephalin (0.1–10 nmol) to rats pretreated with three peptidase inhibitors (Figs. 3 and 4). The $AUC_{0-60\ min}$ values for the antinociceptive action demonstrate that [Met^5]enkephalin-Arg-Gly-Leu is approximately 10-folds more potent than [Met^5]enkephalin in rats pretreated with three peptidase inhibitors (Fig. 4). In the absence of the peptidase inhibitor, however, the antinociceptive potencies of [Met^5]enkephalin-Arg-Gly-Leu and [Met^5]enkephalin were quite slight even at the doses of 10 and 1000 nmol, respectively (Figs. 3 and 4). The potency of [Met^5]enkephalin-Arg-Gly-Leu at the dose of 10 nmol to inhibit the tail-flick response in rats not treated with the peptidase inhibitor was significantly less than that at the dose of 0.01 nmol in rats pretreated with the three peptidase inhibitors (Fig. 4). Additionally, the potency of [Met^5]enkephalin at the dose of 1000 nmol in rats not treated with the peptidase inhibitor was slightly but not significantly smaller than that at the dose of 0.1 nmol, and significantly smaller than that at the dose of 1 nmol, in rats pretreated with the three peptidase inhibitors (Fig. 4).

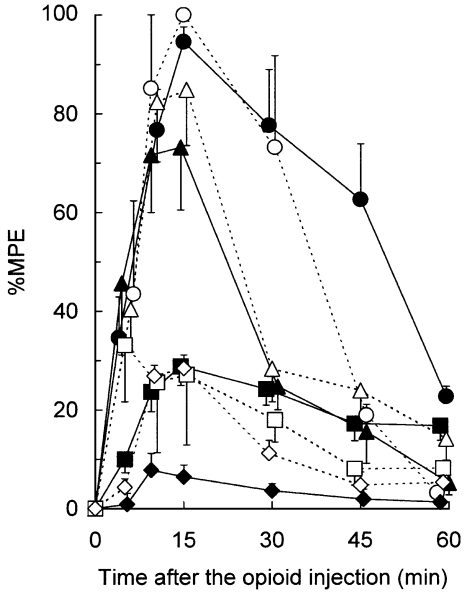

Fig. 3. Comparison of inhibitory effects on the tail-flick response in rats induced by [Met^5]enkephalin-Arg-Gly-Leu with those induced by [Met^5]-enkephalin. [Met^5]enkephalin-Arg-Gly-Leu was given intra-third-ventricularly at doses of 0.01 nmol (■, $n=11$), 0.1 nmol (▲, $n=5$) or 1 nmol (●, $n=9$). [Met^5]enkephalin was given intra-third-ventricularly at doses of 0.1 nmol (□, $n=4$), 1 nmol (△, $n=4$) or 10 nmol (○, $n=4$). The mixture of three peptidase inhibitors, amastatin, captopril and phosphoramidon, at doses of 10 nmol each was given intra-third-ventricularly 10 min before the opioid peptide to all rats except two groups, in which 10 nmol [Met^5]enkephalin-Arg-Gly-Leu (◆, $n=9$) or 1000 nmol [Met^5]enkephalin (◇, $n=5$) was injected intra-third-ventricularly to rats not treated with the peptidase inhibitor. Each symbol represents the mean of n observations. Vertical bars represent the S.E.M.

3.4. Effects of three peptidase inhibitors on the potency of [Met^5]enkephalin-Arg-Gly-Leu in isolated guinea pig ileum and mouse vas deferens

The inhibitory effects of [Met^5]enkephalin-Arg-Gly-Leu on the electrically evoked contractions of both mouse vas

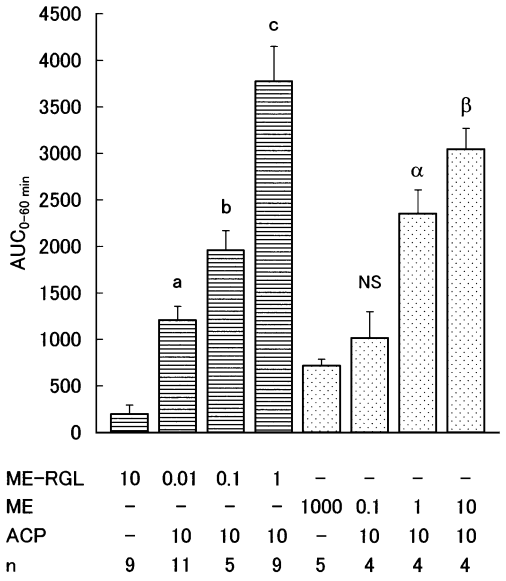

Fig. 4. Dose-dependent antinociceptive effects of [Met^5]enkephalin-Arg-Gly-Leu (ME-RGL) and [Met^5]enkephalin (ME) in rats pretreated with three peptidase inhibitors (ACP), and those in rats not treated with the peptidase inhibitor. The time course of ME-RGL- or ME-induced antinociception in rats expressed as %MPE is shown in Fig. 4 and the $AUC_{0-60\ min}$ values for the antinociceptive action of ME-RGL or ME were calculated for each rat. Each vertical column represents the mean of n observations. Vertical bars represent the S.E.M. $^aP<0.05$, $^bP<0.01$ and $^cP<0.001$ by ANOVA (Scheffe's F-test) when compared with values for a group that had received intra-third-ventricular ME-RGL (10 nmol) alone. $^\alpha P<0.01$ and $^\beta P<0.001$ by ANOVA (Scheffe's F-test) when compared with values for a group that had received intra-third-ventricular ME (1000 nmol) alone. NS, not significant.

Table 1
The enhancing effects of peptidase inhibitors (PIs) on the inhibitory potency of [Met^5]enkephalin-Arg-Gly-Leu in guinea pig ileum (GPI) and mouse vas deferens (MVD)

Preparations	PIs	IC_{50} (nM)	Ratio of potency	Percent difference
GPI	None	142 ± 25	1	76.0 ± 4.3 *
	ACP	32.6 ± 5.6	4.54 ± 0.75	
MVD	None	50.0 ± 5.7	1	77.6 ± 0.87 *
	ACP	11.2 ± 1.3	4.49 ± 0.18	

The mixture of three PIs, amastatin (A), captopril (C) and phosphoramidon (P), at the final concentration of 1 μM each was given 5 min before [Met^5]enkephalin-Arg-Gly-Leu administration. Values are the means ± S.E.M. of four experiments.
* $P < 0.001$.

deferens and the myenteric plexus longitudinal muscle preparation of guinea pig ileum were enhanced approximately 4.5-fold by pretreatment of the preparations with the mixture of three peptidase inhibitors, amastatin, captopril and phosphoramidon, at the concentration of 1 μM each (Table 1).

4. Discussion

Previous reports (Norman and Chang, 1985; Hiranuma et al., 1997) indicated that the captopril-sensitive peptidase plays the most important role among the three peptidases in the hydrolysis of [Met^5]enkephalin-Arg-Gly-Leu. However, the antinociceptive potency of 10 nmol enkephalin octapeptide injected intra-third-ventricularly was not at all augmented by the intra-third-ventricular pretreatment of rats with 10 nmol captopril. This suggests that the two noninhibited peptidases, amastatin- and phosphoramidon-sensitive peptidases, can rapidly and largely inactivate the octapeptide administered intra-third-ventricularly. Additionally, the fact that the antinociceptive action of 1 nmol [Met^5]enkephalin-Arg-Gly-Leu in rats pretreated with the mixture of three peptidase inhibitors, amastatin, captopril and phosphoramidon, was significantly greater than in rats pretreated with any combination of two peptidase inhibitors indicates that any noninhibited peptidase among the three can inactivate significant amounts of [Met^5]enkephalin-Arg-Gly-Leu, which is consistent with previous findings from in vitro experiments (Hiranuma et al., 1997). Furthermore, the rapid inactivation of [Met^5]enkephalin-Arg-Gly-Leu administered intra-third-ventricularly and its prevention by the three peptidase inhibitors were shown in the present investigation by the evidence that the antinociceptive action of 0.01 nmol [Met^5]enkephalin-Arg-Gly-Leu in rats pretreated with the three peptidase inhibitors was significantly greater than that of 10 nmol enkephalin octapeptide in nonpretreated rats.

Our previous study showed that the hydrolysis of [Met^5]-enkephalin-Arg-Gly-Leu by cerebral membrane preparations is almost completely prevented by the presence of the three peptidase inhibitors (Hiranuma et al., 1997). It is not yet known, however, whether or not the hydrolysis of [Met^5]enkephalin-Arg-Gly-Leu in the cerebrospinal fluid is also completely prevented by the three peptidase inhibitors. Low molecular weight opioid peptides such as [Met^5]enkephalin and dynorphin A-(1−7) are shown to be hydrolyzed in cerebrospinal fluid mainly by aminopeptidase M that is inhibited by amastatin, and the other peptidase activities are suggested to be low in cerebrospinal fluid (Benter et al., 1990). Additionally, endopeptidase-24.11, that is inhibited by phosphoramidon, is indicated to play a key role in regulating the concentrations of neuropeptides, including enkephalins, in cerebrospinal fluid (Bourne et al., 1989). Three reports (Benter et al., 1990; Bourne et al., 1989; Hiranuma et al., 1997) suggest that the hydrolysis of [Met^5]enkephalin-Arg-Gly-Leu administered intra-third-ventricularly is largely prevented in the presence of the three peptidase inhibitors: amastatin, captopril and phosphoramidon. Therefore, the antinociceptive potency of [Met^5]enkephalin-Arg-Gly-Leu shown in the present study probably reflects largely the real potency of [Met^5]enkephalin-Arg-Gly-Leu itself.

The involvement of μ-opioid receptors in the antinociceptive action of [Met^5]enkephalin-Arg-Gly-Leu in rats pretreated with the three peptidase inhibitors is suggested by the fact that pA_2 values for naloxone, an opioid antagonist having a preference for μ-opioid receptors (Lord et al., 1977), for enkephalin octapeptide are not significantly different from those for a selective μ-opioid receptor agonist, DAMGO, that were reported previously (Fang et al., 1986; Kitamura et al., 2000). Although [Met^5]enkephalin-Arg-Gly-Leu is known to act on both μ- and δ-opioid receptors (McKnight et al., 1983), the antinociceptive activity of δ-opioid receptor agonists is not likely to be measurable under the present experimental conditions, since the antinociceptive action of [D-Pen^{2,5}]enkephalin (DPDPE), a potent selective δ-opioid receptor agonist, at doses up to 10 nmol could not be detected in our preliminary experiments. These data are in agreement with those of Heyman et al. (1988) showing that the intracerebroventricular administration of DPDPE produces antinociception in the hot-plate, but not in the tail-flick, test with rats. Therefore, only the action of [Met^5]enkephalin-Arg-Gly-Leu at μ-opioid receptors could be estimated using the present antinociceptive test. Interestingly, [Met^5]enkephalin had been shown to be 1.62 times more potent than [Met^5]enkephalin-Arg-Gly-Leu in guinea pig ileum pretreated with the three peptidase inhibitors (Hiranuma et al., 1997), while the antinociceptive action of [Met^5]enkephalin-Arg-Gly-Leu was shown to be approximately 10-folds more potent than [Met^5]enkephalin in rats pretreated with the three peptidase inhibitors. Since both opioid peptides are known to act on μ-opioid receptors (Lord et al., 1977; Hiranuma et al., 1997) in guinea pig ileum, the reason why the μ-opioid receptor-mediated antinociceptive action of [Met^5]enkephalin-Arg-Gly-Leu is significantly greater than that of [Met^5]enkephalin remained to be elucidated in the present study.

The amounts of [Met^5]enkephalin-Arg-Gly-Leu and [Met^5]enkephalin inactivated by the three peptidases during

passage of the peptide from the site of administration to the sites of action (opioid receptors) are likely to be markedly greater in the in vivo experiment than in the in vitro experiment, since the antinociceptive potency of the enkephalin octapeptide or the enkephalin administered intra-third-ventricularly was increased more than 1000- or 10,000-fold by the three peptidase inhibitors, respectively, while the inhibitory potencies of [Met^5]enkephalin-Arg-Gly-Leu in isolated guinea pig ileum and mouse vas deferens were increased approximately 4.5-fold and those of [Met^5]enkephalin in the former and the latter preparations were increased 6.8- and 21-fold by the three peptidase inhibitors, respectively (Kuno et al., 1986).

Since the present and the previous investigations (Taniguchi et al., 1998; Kitamura et al., 2000) show that the antinociceptive potencies of the low molecular weight opioid peptides such as [Met^5]enkephalin, dynorphin-(1–8) and [Met^5]enkephalin-Arg-Gly-Leu, even at the highest dose employed in each experiment, are quite slight, if existent, in rats not treated with the peptidase inhibitor, results of these studies indicate strongly that the intra-third-ventricular pretreatment of rats with the three peptidase inhibitors is essential to determine the precise antinociceptive actions of the low molecular weight opioid peptides administered intra-third-ventricularly. Since the effects of synaptically released short opioid peptides must be significantly potentiated by the prevention of their hydrolytic inactivation, their roles in the central nervous system are anticipated to be more clearly demonstrable in rats pretreated with the three peptidase inhibitors than in rats not treated with the peptidase inhibitor.

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