Full Paper

The Enhancing Effects of Peptidase Inhibitors on the Antinociceptive Action of [Met⁵]Enkephalin-Arg⁶-Phe⁷ in Rats

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Abstract. Previous in vitro studies have shown that the degradation of [Met⁵]enkephalin-Arg⁶-Phe⁷ during incubation with cerebral membrane preparations is largely prevented by a mixture of three peptidase inhibitors: amastatin, captopril, and phosphoramidon. The present in vivo study shows that the inhibitory effect of [Met⁵]enkephalin-Arg⁶-Phe⁷ administered intra-third-ventricularly on the tail-flick response was increased more than 1000-fold by the intra-third-ventricular pretreatment with three peptidase inhibitors. The antinociceptive effect produced by the [Met⁵]enkephalin-Arg⁶-Phe⁷ 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 [Met⁵]enkephalin-Arg⁶-Phe⁷. The present data, together with those obtained from previous studies, clearly show that amastatin-, captopril-, and phosphoramidon-sensitive enzymes play important roles in the inactivation of endogenous opioid peptides, such as [Met⁵]enkephalin, [Met⁵]enkephalin-Arg⁶-Phe⁷, [Met⁵]enkephalin-Arg⁶-Gly⁷-Leu⁸, and dynorphin A (1-8), administered intra-third-ventricularly to rats.

Keywords: [Met⁵]enkephalin-Arg⁶-Phe⁷, opioid peptide, antinociception, peptidase inhibitor

Introduction

It has been shown that when [Met⁵]enkephalin-Arg⁶-Phe⁷ (ME-RF) is incubated with ileal and striatal membrane fractions for 60 min at 37°C in the presence of three peptidase inhibitors (PIs), amastatin (an aminopeptidase inhibitor), captopril (a dipeptidyl carboxypeptidase inhibitor), and phosphoramidon (an endopeptidase-24.11 inhibitor), approximately 87% or more ME-RF remains intact, while in the absence of the peptidase inhibitors, it is completely hydrolyzed during the initial 45-min incubation (1). This shows that ME-RF is hydrolyzed, at least in these membrane preparations, largely by 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 (“enkephali-

nase”, EC 3.4.24.11). Additionally, the potency of ME-RF in isolated guinea-pig ileum preparation has been shown to be significantly increased by amastatin, captopril, or phosphoramidon (1). Thus, it was of interest to examine the in vivo effects of ME-RF on the central nervous system in the presence or the absence of the peptidase inhibitors. In the present investigation, the effects of the three peptidase inhibitors were examined on the antinociception induced by the intra-third-ventricular (i.t.v.) administration of ME-RF. Additionally, it was determined which receptors are concerned with ME-RF-induced antinociception by obtaining the pA₂ values for naloxone, an opioid antagonist having a preference for μ -opioid receptors (2), since ME-RF is known to act on both μ - and δ -opioid receptors (3, 4).

Materials and Methods

Chemicals

Captopril and naloxone hydrochloride were kindly provided by Sankyo (Tokyo). Other chemicals were purchased from the following sources: amastatin and

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phosphoramidon (Peptide Institute, Minoh) and ME-RF (Sigma Japan, Tokyo). 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, maintained at -18°C and then diluted to the desired concentration just before use.

I.t.v. microinjection

Experiments were performed according to the Guidelines for Animal Experimentation in the School of Medicine, Tokai University. Male Wistar rats weighing 180–220 g were anesthetized with pentobarbital sodium (40 mg/kg, i.p.), mounted on a stereotaxic frame, and implanted with stainless-steel injection cannulae (external diameter of 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 (5). The injection cannula was attached to a motor-driven 50- μl microsyringe by polyethylene tubing. PIs or saline were injected 10 min before administration of ME-RF. The volume of solution microinjected was 10 μl for 1 min. The distribution of the drug solution in the cerebroventricular system was verified by infusion of methylene blue dissolved in saline after the experiment.

Tail-flick response

The antinociceptive effect of opioids was measured by the tail immersion assay with 55°C as the nociceptive stimulus (6). The latency to flick the tail from the 55°C water was measured before and 5, 10, 15, 30, 45, and 60 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 percentage of maximal possible effect (%MPE) for each animal at each time was calculated using the following formula: $\% \text{MPE} = [(\text{test latency} - \text{baseline latency}) / (5 - \text{baseline latency})] \times 100$. The AUC (area under the curve) value for the antinociceptive effect of an opioid on each rat was calculated for some experiments.

In vivo apparent pA_2 analysis

Tail-flick latency of rats pretreated with the three PIs was measured before and 15 min after the i.t.v. injection of ME-RF and converted to %MPE. The dose-effect curve of an agonist in each rat was constructed by injecting the rat with agonist at two or three doses, such as 0.5, 1 and 2 nmol, with an inter-injection interval of 48 h. Individual ED_{50} values were calculated by least-squares regression with use of 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 i.t.v. 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 (7).

Statistical analyses

All values are reported as the mean with S.E.M. of the data. A statistical analysis was conducted by using computer software (The SPSS 14.0.1; SPSS, Inc., Chicago, IL, USA) for comparison across the experimental conditions. When a significant difference among the groups of AUC data was obtained in the one-way analysis of variance (ANOVA), the Bonferroni's multiple comparison test or the Dunnett's post-hoc test was applied to define which group contributed to these differences. The level of statistical significance was set at $P < 0.05$.

Results

Effects of PIs on ME-RF-induced inhibition of tail-flick response

Our previous in vivo study showed that the inhibition of the tail-flick response induced by i.t.v. administration of ME was augmented by increasing the doses of the three PIs administered intra-third-ventricularly with the maximum inhibition being attained at the dose of 10 nmol each (8). Furthermore, previous in vitro experiments showed that the concentration of three PIs required to inhibit the hydrolysis of ME was essentially the same as that of ME-RF (1). Therefore, each PI at the dose of 10 nmol was employed to inhibit the targeted peptidase in the present investigation.

As reported previously (8), the PIs by themselves did not significantly change the latency of the tail-flick response. The inhibition of tail-flick response induced by 10 nmol of ME-RF was examined in rats pretreated with the mixture of two or three of the following PIs: amastatin (A), captopril (C), and phosphoramidon (P) at the dose of 10 nmol each (Figs. 1 and 2). The antinociceptive effects of 10 nmol of ME-RF in rats pretreated with three PIs, ACP, was significantly greater than in rats pretreated with any combination of two PIs, AC, AP, or CP (Fig. 2). The $\text{AUC}_{0-60\text{min}}$ values for the antinociceptive effect of ME-RF in rats pretreated with one combination (e.g., AC) of two PIs were not significantly different from those pretreated with any other combination (e.g., AP or CP) of two PIs by Bonferroni's multiple comparison test.

Fig. 1. Effects of the mixture of two or three peptidase inhibitors (PIs), amastatin (A), captopril (C), and phosphoramidon (P), on the ME-RF-induced inhibition of the tail-flick response in rats. The mixture of two or three PIs (AC, $n = 6$; AP, $n = 6$; CP, $n = 6$; ACP, $n = 11$) at the dose of 10 nmol each was given intra-third-ventricularly 10 min before the i.t.v. administration of ME-RF (10 nmol). Vertical bars represent the S.E.M.

Fig. 2. Greater antinociceptive effect of ME-RF in rats pretreated with the mixture (ACP) of three peptidase inhibitors (PIs), amastatin (A), captopril (C), and phosphoramidon (P), than in those pretreated with any combination of two PIs (AC, AP, or CP). The time-course of ME-RF-induced antinociception in rats expressed as %MPE is shown in Fig. 1. The AUC_{0-60min} values for the antinociceptive effect of ME-RF were calculated for each rat. Vertical bars represent the S.E.M. Significantly different from the values of the group that received three PIs (ACP), determined by Bonferroni's multiple comparison test following one-way ANOVA: *** $P < 0.001$.

The effects of the i.t.v. administration of 0.01, 0.1, 1, and 10 nmol of ME-RF in rats pretreated with three PIs at the dose of 10 nmol each were investigated (Figs. 3 and 4). The potency of ME-RF at the dose of 10, 1, or 0.1 nmol in rats pretreated with three PIs was significantly greater than that of ME-RF at the dose of 10 nmol in rats not treated with PIs (Fig. 4). The potency of ME-RF at the dose of 10 nmol in rats not treated with PIs was

Fig. 3. The time course of change in ME-RF-induced antinociceptive effects in rats pretreated with the mixture (ACP) of three peptidase inhibitors (PIs), amastatin (A), captopril (C), and phosphoramidon (P). The first group of rats, which was not treated with PIs, received intra-third-ventricularly ME-RF (10 nmol) ($n = 6$). In the second to fifth group of rats, a mixture of three PIs (ACP) at a dose of 10 nmol each was injected intra-third-ventricularly 10 min before the i.t.v. injection of ME-RF (0.01 nmol, $n = 5$; 0.1 nmol, $n = 9$; 1.0 nmol, $n = 18$; 10 nmol, $n = 11$). ME-RF-induced antinociceptive effects in rats were expressed as %MPE. Vertical bars represent the S.E.M.

Fig. 4. The AUC_{0-60min} values for the ME-RF-induced antinociceptive effects, expressed as %MPE in Fig. 3, in rats pretreated with the mixture (ACP) of three peptidase inhibitors (PIs): amastatin (A), captopril (C), and phosphoramidon (P) at the dose of 10 nmol each. The AUC_{0-60min} values for the antinociceptive effect of ME-RF were calculated for each rat. Vertical bars represent the S.E.M. Significantly different from the values of a group that received 10 nmol ME-RF alone by Dunnett's post-hoc test following one-way ANOVA: ** $P < 0.01$, *** $P < 0.001$; N.S., not significant.

slightly but not significantly smaller than that at the dose of 0.01 nmol in rats treated with three PIs (Fig. 4). Thus, the antinociceptive effect of ME-RF administered i.t.v. on the tail-flick response was increased more than 1000-fold by the i.t.v. administration of three PIs.

Fig. 5. Schild plots obtained for naloxone in the antinociception assay of ME-RF. 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 each panel. The 95% confidence limits are shown in parentheses.

Effects of naloxone on ME-RF-induced inhibition of tail-flick response

The dose-effect curves for ME-RF were shifted dose-dependently to the right after pretreatment subcutaneously with various doses of naloxone (0.01, 0.02, and 0.05 mg/kg) 5 min before the i.t.v. administration of ME-RF. Figure 5 shows the Schild plots for naloxone with values derived from individual dose ratios for each rat. The pA_2 value for naloxone against [D-Ala², N-Me-Phe⁴, Gly-ol]enkephalin (DAMGO), a selective μ -agonist, under the same experimental condition, was reported previously (9); the reported pooled pA_2 values and slopes (95% confidence limits shown in parentheses) were 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. 5 demonstrate that the effectiveness of naloxone to antagonize the antinociceptive effects of ME-RF is similar to and not significantly different from that of DAMGO reported previously (9).

Discussion

The fact that the antinociceptive action of 10 nmol ME-RF in rats pretreated with the mixture of three PIs, amastatin, captopril, and phosphoramidon, was significantly greater than in rats pretreated with any combination of two PIs indicates that any non-inhibited peptidase among the three can inactivate significant amounts of

ME-RF, which is consistent with previous findings from an in vitro experiment (1). Additionally, the rapid inactivation of ME-RF administered i.t.v. and its prevention by the three PIs were shown in the present investigation by the evidence that the antinociceptive action of 0.1 nmol ME-RF in rats pretreated with the three PIs was significantly greater than that of 10 nmol enkephalin heptapeptide in non-pretreated rats.

The previous study (1) showed that inactivation of ME-RF was caused by splitting of the Tyr¹-Gly² bond and Gly³-Phe⁴ bond by amastatin- and phosphoramidon-sensitive enzymes, respectively, and by sequential splitting of the Met⁵-Arg⁶ bond and Gly³-Phe⁴ bond by captopril-sensitive dipeptidyl carboxypeptidase I. Our previous study showed that the hydrolysis of ME-RF by cerebral membrane preparations is largely prevented by the presence of the three PIs (1). It is not yet known, however, whether or not the hydrolysis of ME-RF in the cerebrospinal fluid is also completely prevented by the three PIs. Low molecular weight opioid peptides such as [Met⁵]enkephalin (ME) 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 (10). Additionally, endopeptidase-24.11, which is inhibited by phosphoramidon, is indicated to play a key role in regulating the concentrations of neuropeptides, including enkephalins, in cerebrospinal fluid (11). Three reports (1, 10, 11) suggested that the hydrolysis of ME-RF administered intra-third-ventricularly is largely prevented in the presence of the three PIs: amastatin, captopril, and phosphoramidon. Therefore, the antinociceptive potency of ME-RF shown in the present study probably largely reflects the real potency of ME-RF itself.

The involvement of μ -opioid receptors in the antinociceptive effect of ME-RF in rats pretreated with the three PIs is suggested by the fact that the pA_2 values for naloxone, an opioid antagonist having a preference for μ -opioid receptors (2), for the ME-RF are not significantly different from the previously reported values for the selective μ -opioid receptor agonist DAMGO (9, 12). Although ME-RF is known to act on both μ - and δ -opioid receptors (3, 4), the antinociceptive effect of δ -opioid receptor agonists is not likely to be measurable under the present experimental conditions, since the antinociceptive effect 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. (13) 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 ME-RF at μ -opioid receptors could be estimated using the present antinociceptive test.

Since the present and the previous investigations (8, 9, 14) show that the antinociceptive potencies of the low molecular weight opioid peptides such as ME, [Met⁵]enkephalin-Arg⁶-Gly⁷-Leu⁸, dynorphin A (1-8), and ME-RF, even at the highest dose employed in each experiment, are only slight, if existent, in rats not treated with the PIs, results of these studies strongly indicate that the i.t.v. pretreatment of rats with the three PIs is essential to determine the precise antinociceptive effects 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 PIs than in rats not treated with the PIs.

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