2S,3R 3-Amino-2-Hydroxy-4-Phenylbutanoic Acid Derivatives, Enkephalinase Inhibitors, Augment Met$^5$-Enkephalin-Induced Antinociception

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Abstract—It has been accepted that the periaqueductal gray matter of the mid brain (PAG) and the reticular formation of the medulla oblongata have antinociceptive roles in the pain control pathways of mammals, and met$^5$-enkephalin may act as one of the pain control substances in those regions. In the present study, the effects of 2S,3R 3-amino-2-hydroxy-4-phenylbutanoic acid (AHPA) derivatives on met$^5$-enkephalin-induced antinociception were examined by a hot plate method in mice. The elevation of pain threshold induced by an intracisternal administration of met$^5$-enkephalin was enhanced by AHPA derivatives. The rank order of potency for these agents was as follows: bestatin>D-Phe-AHPA>AHPA-D-Ala>p-OH-AHPA-D-Phe>AHPA. This order was roughly correlated to that of the enkephalinase inhibitory activity of the AHPA derivatives. These results indicate that the inhibition of enkephalinase may produce the augmentation of the exogenous met$^5$-enkephalin-induced antinociception. It is also suggested that AHPA derivatives may cause the enhancement of the endogenous met$^5$-enkephalin-mediated antinociception.

It has been generally accepted that endogenous opioid peptides such as $\beta$-endorphin and met$^5$-enkephalin are "the endogenous pain control substances" (1). Since the periaqueductal gray matter (PAG) contains a large number of opioid receptors (2) and the tissue level of enkephalins is higher in this region in accordance with the higher receptor distribution (3, 4), most investigators agree that PAG has an important role as a site of antinociception. Takagi et al. (5) and Akaike et al. (6) reported that the sites for the antinociceptive action of morphine are localized mainly in the nucleus reticularis gigantocellularis (NRGC) and the nucleus reticularis paragigantocellularis (NRPG) of the medulla oblongata. Therefore, the reticular formation of the medulla oblongata may also be regarded as an important site of antinociception.

When enkephalins were treated with brain homogenate, various fragments from enkephalins were obtained, suggesting that several enzymes may be involved in the degradation of enkephalins in situ. Enkephalinase or dipeptidyl carboxypeptidase is one of these enkephelin-degrading enzymes (7–9). Thus, the application of enkephalinase inhibitors should augment antinociception if it is mediated by enkephalins. 2S,3R 3-Amino-2-hydroxy-4-phenylbutanoic acid (AHPA) derivatives were found to be enkephalinase inhibitors (10). In the present study, we examined the effect of intracisternally injected AHPA derivatives, enkephalinase inhibitors, on met$^5$-enkephalin-induced antinociception, since intracisternally injected drugs can easily reach broad areas of the brain stem including the PAG and aqueduct of the midbrain and the NRGC and NRPG of the medulla oblongata (11).
Table 1. Chemical structures of 2S,3R 3-amino-2-hydroxy-4-phenyl butanoic acid (AHPA) derivatives

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\begin{array}{ccc}
\text{derivatives} & R_1 & R_2 \\
\text{AHPA} & H & OH \\
p-OH-AHPA-D-Phe & H & D-Phe \\
D-Phe-AHPA & D-Phe & OH \\
AHPA-D-Ala & H & D-Ala \\
AHPA-L-Leu (bestatin) & H & L-Leu \\
\end{array}
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Amino acids are represented by the following three letter symbols: D-Phe: D-phenylalanine, D-Ala: D-alanine, L-Leu: L-leucine.

Materials and Methods

Materials: 2S,3R 3-Amino-2-hydroxy-4-phenylbutanoic acid (AHPA), AHPA-D-Ala, p-OH-AHPA-D-Phe, D-Phe-AHPA, AHPA-D-Arg and AHPA-L-Leu (bestatin) (Table 1) were provided by Meiji Seika Co., Ltd. (Yokohama). Met\textsuperscript{5}-enkephalin was purchased from Peptide Institute, Inc. (Minoo, Osaka) and brilliant blue from Tokyo Kasei Co. (Tokyo). The purified water used in this study was prepared by a Milli Q Water Purification System (Millipore Co., Bedford, MA).

Animals and experimental procedures: Male ddY mice, weighing 20 to 30 g, were used. Pain thresholds of mice were measured by using the hot plate method of Woolfe and MacDonald (12). The hot plate was maintained at a temperature of 55.5 to 56.0°C by using a thermistor. For the purpose of determining the pain threshold of mice or the latency, the length of time required for mice placed on the hot plate to initiate any one of the following behaviors: jumping or shaking, licking, holding or lifting their paws was measured in a room kept at 24±1°C and at 55±5% relative humidity and protected from external noise (13). The cut-off time was set at 25 sec in order to protect mice from burning their paws. Prior to the experiments, mice were tested twice for the latency, and those showing a latency ranging from 2 to 7 sec were used.

AHPA derivatives and met\textsuperscript{5}-enkephalin were dissolved in purified water, and a 10 μl aliquot of the aqueous solution of met\textsuperscript{5}-enkephalin, AHPA derivatives or a mixture of them was injected slowly into the cisterna magna by the method of Ueda et al. (11). AHPA derivatives were used at the concentration of 3 μg/μl, since doses of AHPA derivatives higher than 30 μg could not be dissolved in 10 μl of water. Distribution of the injected substances was checked by dissolving 30 μg of brilliant blue in 10 μl of the aqueous solution containing met\textsuperscript{5}-enkephalin or AHPA derivatives.

The pain thresholds of mice were measured at 10 min before, immediately before and at 10, 20, 30, 45 and 60 min after the intracisternal injection.

Results

Since the effect of met\textsuperscript{5}-enkephalin is reported to be short in duration and low in efficacy (11), we could not detect the increase of the latency at the dose of 70 μg/mouse in some instances (Figs. 1, 3 and 5). AHPA (30 μg/mouse) revealed no significant increase in the latency by itself, and it could not significantly augment the enkephalin-induced antinociception 10 min after the intracisternal injection (Fig. 1). p-OH-AHPA-D-Phe (30 μg/mouse) increased the latency at 10 min after its injection, and it slightly enhanced the enkephalin-induced antinociception (P<0.1) (Fig. 2). D-Phe-AHPA (30 μg/mouse) increased the latency during a period of 10 to 20 min after its injection and significantly enhanced the enkephalin-induced antinociception 10 min after the in-
AHPA-D-Ala (30 μg/mouse) revealed significant antinociception during a period of 10 to 20 min after the injection, but failed to enhance the enkephalin-induced antinociception (Fig. 4). AHPA-D-Ala caused a sedation of mice, and the sedative effect was augmented by the concomitant administration of met⁵-enkephalin which caused no sedation by itself.

Bestatin (30 μg/mouse) significantly increased the latency during a period of 10 to 20 min after the injection and enhanced the met⁵-enkephalin-induced antinociception (Fig. 5). Bestatin showed a slight sedative effect which was augmented by met⁵-enkephalin. The concomitant injection of bestatin and met⁵-enkephalin tended to lower the skeletal muscle tone of mice during a period of 10 to 20 min after their injection.

Of course, antinociceptive effects of the concomitant use of met⁵-enkephalin with AHPA derivatives were effective and statistically significant (P<0.05 for AHPA and p-OH-AHPA-D-Phe and P<0.01 for D-Phe-AHPA, AHPA-D-Ala, and bestatin at 10 min after the injection).

**Discussion**

Various methods such as the hot plate method, tail-pinch method and tail flick method have widely been used for the measurement of analgesic activity, and each of them has been claimed to provide an individual, characteristic parameter for the evaluation of analgesia. For example, the tail flick method is due to a pure spinal reflex (14). In tail-pinch method, the animals tend to develop a delay in response to stimulations after repeated testings because of the damage to their tails (15). Ueshima (16) reported that the hot plate method is attributed to centers located higher than the level of the spinal cord. We believe that the drugs such as met⁵-enkephalin and AHPA derivatives injected into the cisterna magna may act on the upper brain rather than the spinal cord. Therefore,
we adopted the hot plate method which is less affected by the spinal reflex.

It is well-known that various opioid peptides possess antinociceptive activity. Among the endogenous opioid peptides, met\textsuperscript{5}-enkephalin has been presumed to be a putative inhibitory neurotransmitter in the central nervous system (17). However, met\textsuperscript{5}-enkephalin could not induce antinociception when injected peripherally and caused it only when injected into the brain (11), but the central administration lasted only 10 min or so. These findings suggest rapid degradation of met\textsuperscript{5}-enkephalin by enzymes. Enzymes which are known to decompose enkephalins are as follows: aminopeptidase which cleaves the Tyr\textsuperscript{1}-Gly\textsuperscript{2} bond of enkephalins, dipeptidyl aminopeptidase which splits the Gly\textsuperscript{2}-Gly\textsuperscript{3} bond, dipeptidyl carboxypeptidase (enkephalinase) and angiotensin converting enzyme (ACE) which cleave the Gly\textsuperscript{3}-Phe\textsuperscript{4} bond, and carboxypeptidase which splits the Phe\textsuperscript{4}-Met\textsuperscript{5} bond (1). It has also been reported that the distribution of enkephalinase was as-
associated with that of the opioid receptors in the PAG (18). Hachisu et al. (10) reported that AHPS derivatives inhibit enkephalinase. In the present study, we could not detect anything more than a weak antinociceptive activity by the respective injections of AHPS derivatives or met^5^-enkephalin, with the exception of bestatin injection, but could produce statistically significant increases in the latency by the concomitant injection of met^5^-enkephalin with AHPS derivatives. Therefore, we would like to consider that concomitant injections of AHPS derivatives with met^5^-enkephalin may produce genuine antinociception, and the enhancement of met^5^-enkephalin-induced antinociception by bestatin may be related to the inhibitory action of bestatin on aminopeptidase. In the experiment of Waksman et al. (22), however, bestatin could also inhibit enkephalinase, because the increase of the concentration of the substrate induced by its inhibitory effects on aminopeptidase might hide its apparent inhibition on enkephalinase.

Aoki et al. (23) reported that there are several kinds of aminopeptidases, and the most likely aminopeptidase involved in the degradation of met^5^-enkephalin may be membrane-bound aminopeptidase. AHPS derivatives used in our experiments besides bestatin have been reported to possess no inhibitory action on membrane-bound aminopeptidase (20). However, the results obtained in our experiments on the AHPS derivatives cannot be explained on the basis of their enkephalinase inhibition alone.

Although bestatin tended to produce muscle relaxation by the concomitant injection with met^5^-enkephalin, we can consider that the muscle relaxation is so weak that the weak muscle relaxation may be able to augment the met^5^-enkephalin-induced antinociception in the tail flick test but is unable to do so in our hot plate method.

These results suggest that met^5^-enkephalin-induced antinociception is enhanced by AHPS derivatives, which possess an inhibitory effect on enkephalinase, and they also suggest that AHPS derivatives may enhance the antinociception if endogenous met^5^-enkephalin is involved in the production of antinociception.

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Waksman et al. (22) reported that bestatin decreased the contents of $^3$H-Tyr, but increased those of $^3$H-Tyr-Gly-Gly in the incubation of $^3$H-met^5^-enkephalin with rat brain striatal slices. Therefore, the enhancement of met^5^-enkephalin-induced antinociception by bestatin may be related to the inhibitory action of bestatin on aminopeptidase. In the experiment of Waksman et al. (22), however, bestatin could also inhibit enkephalinase, because the increase of the concentration of the substrate induced by its inhibitory effects on aminopeptidase might hide its apparent inhibition on enkephalinase.
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