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PURIFICATION AND IDENTIFICATION OF ENDOGENOUS ANTI-OPIOID SUBSTANCES FROM BOVINE BRAIN

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SUMMARY: Two fractions with high potency for reversing the inhibitory effect of Met-enkephalin on the electrically induced contractility of guinea-pig ileum have been purified from bovine brain extract. Unexpectedly, one isolated peptide was identified as [Val^5]-angiotensin I and the other fraction was [Val^5]-angiotensin II, as judged by chromatographic comparisons on HPLC and amino acid analysis. Since angiotensins did not affect opioid binding to brain membrane, we consider that angiotensins may act as physiological antagonists to the opioid system in the brain, as well as in the guinea-pig ileum.

Since the discovery of opioid peptides in the brain, the existence of an endogenous anti-opioid system has been suggested (1-3). Moreover, several known peptides i.e. melanotropin release inhibiting factor (4), ACTH fragment (5), β-endorphin fragment (6), and CCK-8 (7) possess anti-analgesic properties *in vivo*. However, none of these studies performed actual isolation of anti-opioid substances from the brain.

In our laboratory, two novel analgesic peptides, kyotorphin (8) and neo-kyotorphin (9), were previously isolated and identified from the bovine brain. In our search for unknown anti-opioid substances, two fractions obtained from the bovine brain extract were found to antagonize the effect of Met-enkephalin on the guinea-pig ileum, *in vitro*. Unexpectedly, they were identified as [Val^5]-angiotensin I and II. We report herein the isolation procedures and preliminary pharmacological experiments done to re-evaluate angiotensin as an endogenous opioid antagonist.

MATERIALS AND METHODS

MATERIALS: Fresh bovine brains minus cerebella, purchased from a local slaughter house within 30min after killing, were washed thoroughly in ice-cold saline to remove all blood, then minced and rapidly frozen in dryice-acetone (-80°C).

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All synthetic peptides were from the Protein Research Foundation, Osaka, Japan. Captopril, a selective angiotensin converting enzyme (EC 3.4.15.1) inhibitor, was from Sankyo Co., Ltd. Naloxone was a gift from Endo Laboratories. Reverse phase HPLC columns, Cosmosil 5C18 (4.6X150mm) and 5CN-R (4.6X150mm), were purchased from Nakarai Chemicals Co., Ltd., Kyoto, Japan.

BIOASSAY: The electrically stimulated myenteric plexus-longitudinal muscle preparations of guinea-pig ileum (10) were used. Opioid antagonistic activity of each sample was evaluated as the potency required to shift the dose-response curve of Met-enkephalin to the right when a sample was injected about 4min before or after the injection of Met-enkephalin.

PURIFICATION: Purification methods are given in "RESULTS". All chromatographic steps with gel columns were performed at 4°C, except for HPLC which was carried out at room temperature. Eluates from the columns were monitored by measuring absorbance at 280nm or 210nm.

AMINO ACID ANALYSIS: Amino acid composition of the isolated peptide was determined using a Hitachi-835 amino acid analyser, after hydrolysis of the peptide (ca.1nmol) in 6N-HCl containing 0.1% phenol at 105°C for 18hr. N-terminal determination was performed by the dansyl method (11). The dansyl amino acids were identified by HPLC, on a subnanomole scale (12).

OPIOID RECEPTOR BINDING ASSAY: Brain membranes were prepared from male Sprague-Dawley rats, male white guinea pigs, and male white rabbits. Binding assay was carried out at 0°C for 3hr, as previously reported (13). Used labelled ligands were [^{3}H]-naloxone, [^{3}H]-D-Ala^2,D-Leu^5-enkephalin, and [^{3}H]-dynorphin(1-9) as μ-, δ-,and κ- opioid receptor ligand, respectively.

RESULTS

EXTRACTION AND PURIFICATION: Fig.1 shows an outline of the purification procedure. Frozen fragments of 100 bovine brains (40kg) were homogenized in 200 liters of acetone with a Polytron homogenizer at -10°C. Residue of filtrated homogenate was dried to fine powder (7.5kg), and the powder was

100 bovine brains (40kg)
↓
acetone powder (7.5kg)
↓
1M CH_3COOH + 20mM HCl extract
↓
SP-Sephadex C-25 batch neutral fraction
↓
Sephadex G-25 Vo X 1.6-2.2 (46g)
↓
CM-Sephadex C-25 gradient peak B (2.1g)
↓
Bio-Gel P-2 Vo X 2.0-2.3 (200mg)
↓
Sephadex G-15 Vo X 1.2-1.6 (117mg)
↓
HPLC [Cosmosil 5CN-R]
↓
HPLC [Cosmosil 5C18]
↓
HPLC [Cosmosil 5C18]
↓
pure peptide (10nmol)

Fig.1: Procedure used to purify the anti-opioid substances from bovine brain.

extracted twice with 20 liters of 1M CH_3COOH + 20mM HCl for 18hr at 4 °C. After centrifugation, the supernatant was adjusted to pH3.5 with NaOH and treated batch-wise with SP-Sephadex C-25. Adsorbed material, which was eluted at pH9.0, was lyophilized and applied to a column (10X100cm) of Sephadex G-25 (medium) equilibrated with 0.5% HCOOH + 0.5% pyridine. The opioid antagonistic activity emerged from a small peptide fraction in a range of 1.6-2.2 X void volume. This active fraction was lyophilized (46g), dissolved in 0.05M $[Na^+]$-acetate buffer pH5.5 containing 5% n-propyl alcohol (added in order to prevent non-specific adsorption of peptides to glass-wall), and loaded on a CM-Sephadex C-25 column (5X50cm). Two active fractions obtained with a linear gradient elution (Fig.2) antagonized Met-enkephalin in the guinea-pig ileum, in a similar manner. Because of a larger amount of activity, peak B was further purified by gel filtration with Bio-Gel P-2 (200-400mesh, 5.5X90cm, 2.5% HCOOH + 5% n-propyl alcohol) and Sephadex G-15 (1.8X160cm, 3% HCOOH + 30% n-propyl alcohol), successively. The final three steps of purification

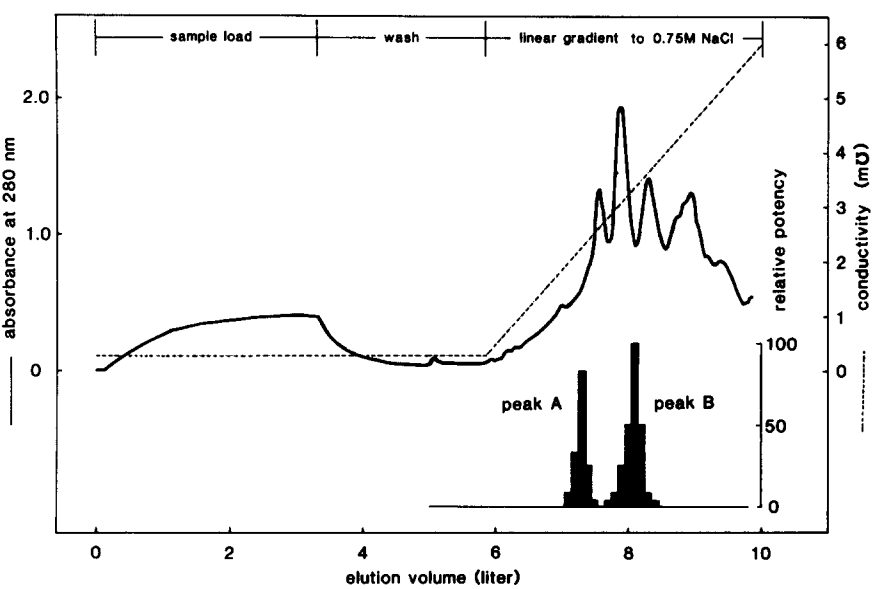

Fig.2: CM-Sephadex C-25 ion exchange chromatography of the small peptide fraction obtained from Sephadex G-25.
Column size: 5 X 50 cm. Flow rate: ca. 200 ml/hr.
Buffer: 0.05M $[Na^+]$-acetate pH5.5 containing 5% n-propyl alcohol.
Anti-opioid activity is shown by the vertical hatched bar as relative potency (maximum fraction = 100).

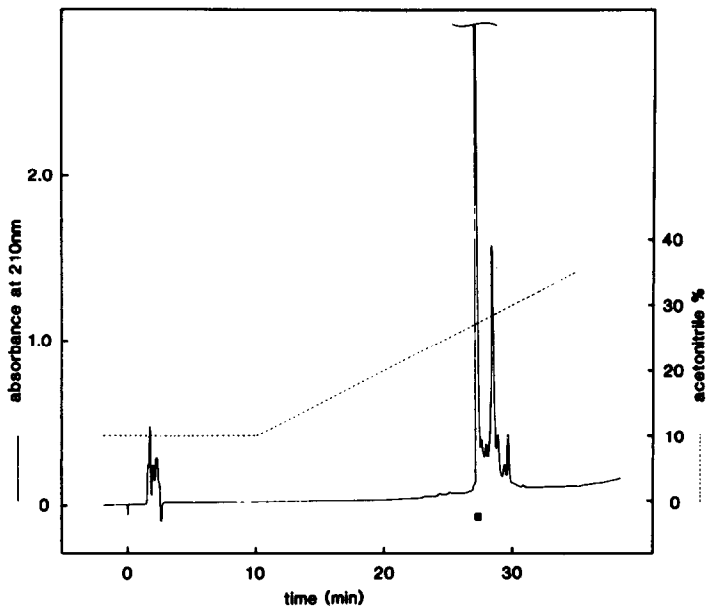

Fig.3: Final purification of the peak B-derived peptide by HPLC. Column: Cosmosil 5C18 (4.6X150mm). Flow rate: 1.0 ml/min. Buffer: 5mM potassium phosphate pH3 + 10mM Na_2SO_4. Anti-opioid activity was observed at the hatched area.

were achieved by HPLC, first with Cosmosil 5CN-R (35min gradient of acetonitrile from 10 to 35% in 5mM phosphate buffer pH3 + 10mM Na_2SO_4), then Cosmosil 5C18 (35min gradient of acetonitrile from 10 to 35% in 0.1% TFA), and finally Cosmosil 5C18 again (Fig.3).

IDENTIFICATION: Amino acid composition of the isolated peptide determined after hydrolysis was found to be: Asp_1, Val_2, Leu_1, Tyr_1, Phe_1, His_2, Arg_1, Pro_1. The N-terminal of the peptide was identified with Asp by the dansyl method. These results suggested that the isolated peptide might be [Val^5]-angiotensin I (Asp-Arg-Val-Tyr-Val-His-Pro-Phe-His-Leu), one of the natural occurring forms of bovine angiotensins. Therefore, we compared the retention time (Rt) of the isolated peptide with that of synthetic [Val^5]-angiotensin I. Although desalted material derived from peak A was not isolated, it was also examined using HPLC by the same methods. Table 1 shows that the Rt's of the isolated peptide from peak B and the active substance in peak A are precisely the same as those of [Val^5]-angiotensin I and [Val^5]-angiotensin II, respectively, even under four different conditions of HPLC.

Table I

Retention times of various synthetic peptides and the purified peptides under four different conditions with two reverse phase HPLC columns.

column	Cosmosil 5CN-R		Cosmosil 5C18	
buffer	5mM PB pH3 +10mM Na_2SO_4	0.1% TFA	5mM PB pH3 +10mM Na_2SO_4	0.1% HFBA
CH_3CN	0-10min 10% -35min -35%	0-10min 10% -35min -35%	0-10min 10% -35min -35%	28% constant
bombesin	25.7	30.0	30.0	11.7
bradykinin	11.2	----	23.2	14.8
CCK-8	32.9	----	----	8.7
LH-RH	20.8	26.6	23.5	9.6
neurotensin	23.9	30.8	28.3	27.1
somatostatin	30.5	35.4	----	24.2
substance P	25.4	31.2	32.2	22.8
[Ile^5]-angiotensin I	22.7	32.2	----	35.4
angiotensin II	16.2	29.8	26.5	18.0
[Val^5]-angiotensin I	20.2	30.4	27.5	30.4
angiotensin II	10.1	28.0	23.8	15.6
isolated peptide	20.2	30.4	27.5	30.4
peak A activity	10.1	28.0	23.8	15.6

The peak of each peptide was detected at 210nm. Flow rate: 1.0 ml/min. PB; potassium phosphate buffer. TFA; trifluoroacetic acid. HFBA; heptafluoro butyric acid.

EVALUATION OF ANTI-OPIOID EFFECTS OF SYNTHETIC ANGIOTENSINS: Synthetic angiotensins showed the same anti-opioid activities as the purified peptides. Fig.4a illustrates a representative effect of the isolated peptide from peak B ([Val^5]-angiotensin I) and synthetic [Val^5]-angiotensin II. [Val^5]-angiotensin II shifted the dose-response curve of Met-enkephalin to the right in a parallel manner. The effect of [Val^5]-angiotensin I was almost equi-potent to [Val^5]-angiotensin II , but was abolished by pretreatment with Captopril (Fig.4b).

OPIOID RECEPTOR BINDING ASSAY: Neither [Val^5]-angiotensin I nor [Val^5]-angiotensin II (up to 10^{-6}M) significantly decreased the μ-, δ-, or κ-opioid binding to any of the brain membrane preparations (data not shown).

DISCUSSION

This is the first successful isolation of angiotensin from bovine brain as well as the first attempt to isolate the anti-opioid substance. We consider

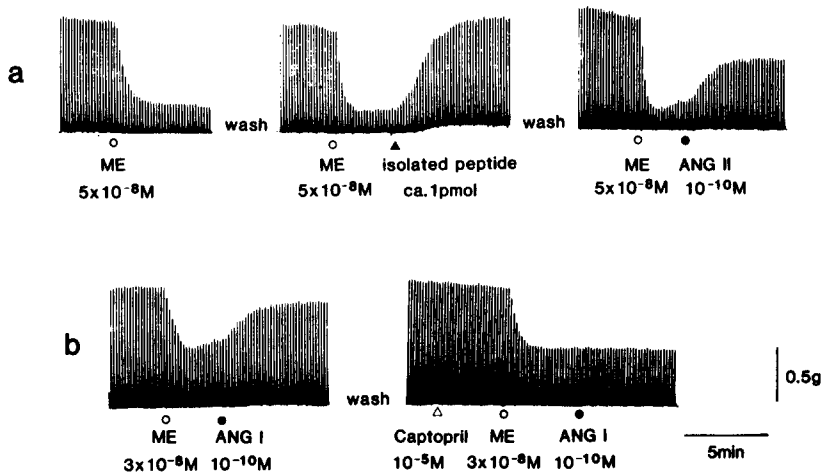

Fig.4: Antagonism of the isolated peptide and the synthetic angiotensins to the inhibitory effect of Met-enkephalin on the guinea-pig ileum myenteric plexus-longitudinal muscle preparations. The preparations were stimulated coaxially at 0.1Hz, 0.6msec-duration, 100V. Each drug concentration is represented as a final concentration in an organ bath (1.5ml). ME; Met-enkephalin. AGT I; [Val^5]-angiotensin I. AGT II; [Val^5]-angiotensin II.

the obtained angiotensins were neurogenic peptides, not derived from blood or artifacts, for the following reasons: First, the amount of purified [Val^5]-angiotensin I, estimated to be 10nmol from the result of amino acid analysis, and that of II are too large to be derived from contamination of blood, because normal concentrations of immuno-reactive angiotensin in the blood are less than 100 pico moles per liter (14). Secondly, the same fractions of angiotensin I and II were obtained when bovine brains were extracted with boiling 1M CH_3COOH + 20mM HCl for 10min to denaturalize proteolytic enzymes, in our preliminary studies (data not shown).

[Val^5]-angiotensin I and II had potent anti-opioid activities in the guinea-pig ileum. The finding that Captopril abolished the effect of [Val^5]-angiotensin I suggests that [Val^5]-angiotensin II is the active form of angiotensins. Since angiotensins did not affect any sub-type of opioid receptor binding to the brain membrane, the antagonism of angiotensins to the opioid system is probably physiological, not receptor-competitive. The relations between the angiotensins and the opioid peptides in the central nervous system are poorly understood (15).

Recently it was demonstrated that central actions of angiotensin were inhibited by morphine and opioid peptides (16,17). Vice versa, in our preliminary experiments, we obtained evidence that intracerebroventricular administration of angiotensin II inhibits the morphine-induced analgesia in mice. The details on anti-analgesic activities of angiotensins will be reported elsewhere.

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