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EXISTENCE OF MET-ENKEPHALIN-ARG^6-GLY^7-LEU^8 WITH MET-ENKEPHALIN, LEU-ENKEPHALIN AND MET-ENKEPHALIN-ARG^6-PHE^7 IN THE BRAIN OF GUINEA PIG, RAT AND GOLDEN HAMSTER

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High performance liquid chromatography (HPLC) coupled with specific radioimmunoassays for methionine-enkephalin-Arg^6-Gly^7-Leu^8 (Met-E-Arg^6-Gly^7-Leu^8), methionine-enkephalin (Met-E), leucine-enkephalin (Leu-E) and methionine-enkephalin-Arg^6-Phe^7 (Met-E-Arg^6-Phe^7) has demonstrated that Met-E-Arg^6-Gly^7-Leu^8 exists together with Met-E, Leu-E and Met-E-Arg^6-Phe^7 in the brain of guinea pig, rat and golden hamster. The content of Met-E-Arg^6-Gly^7-Leu^8 was comparable to those of Leu-E and Met-E-Arg^6-Phe^7, whereas that of Met-E was the highest among the four opioid peptides. These results are compatible with the recent studies on the nucleotide sequence of cloned cDNA for preproenkephalin from bovine adrenal medulla, which reveal that this precursor molecule contains four copies of Met-E and one copy each of Leu-E, Met-E-Arg^6-Phe^7 and Met-E-Arg^6-Gly^7-Leu^8. The co-existence of Met-E-Arg^6-Gly^7-Leu^8 with Met-E, Leu-E and Met-E-Arg^6-Phe^7 suggests that their biosynthetic pathway in the brain is similar to that in the adrenal medulla.

INTRODUCTION

Methionine-enkephalin-Arg^6-Gly^7-Leu^8 (Met-E-Arg^6-Gly^7-Leu^8) is a newly discovered endogenous opioid peptide from bovine adrenal chromaffin granules (1) and has proved to be contained in the preproenkephalin molecule together with methionine-enkephalin (Met-E), leucine-enkephalin (Leu-E) and methionine-enkephalin-Arg^6-Phe^7 (Met-E-Arg^6-Phe^7) as revealed by analysis of the nucleotide sequence of cloned cDNA for preproenkephalin from bovine adrenal medulla (2,3). To elucidate whether or not Met-E-Arg^6-Gly^7-Leu^8 is

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Abbreviations used: Met-E, Tyr-Gly-Gly-Phe-Met; Leu-E, Tyr-Gly-Gly-Phe-Leu; Met-E-Arg^6-Gly^7-Leu^8, Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu; Met-E-Arg^6-Phe^7, Tyr-Gly-Gly-Phe-Met-Arg-Phe; HPLC, high performance liquid chromatography; RIA, radioimmunoassay; ODS, octadecylsilane; LI, like immunoreactivity.

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present in the brain, we have examined brain extracts of guinea pigs, rats and golden hamsters using high performance liquid chromatography (HPLC) coupled with specific radioimmunoassays (RIAs) for Met-E-Arg^6-Gly^7-Leu^8, Met-E, Leu-E and Met-E-Arg^6-Phe^7.

MATERIALS AND METHODS

Peptides

Met-E-Arg^6-Gly^7-Leu^8 was synthesized by a conventional solution method. The homogeneity of the synthetic peptide was confirmed by thin-layer chromatography, HPLC and paper electrophoresis. Met-E-Arg^6-Phe^7 and dynorphin were generously provided by Drs. M. Fujino and M. Wakimasu, Central Research Division, Takada Chemical Industries, Ltd., Osaka, Japan. Met-E and Leu-E were provided by Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan. α-Neo-endorphin and β-neo-endorphin were donated by Dr. H. Matsuo, Miyazaki Medical College, Miyazaki, Japan. Human β-endorphin was a gift from Dr. C.H. Li, University of California, San Francisco, U.S.A.. Human ACTH was supplied by National Pituitary Agency, U.S.A..

Conjugation of Met-E-Arg^6-Gly^7-Leu^8 and Met-E-Arg^6-Phe^7

Met-E-Arg^6-Gly^7-Leu^8 and Met-E-Arg^6-Phe^7 were conjugated to bovine thyroglobulin (Sigma, St. Louis, Missouri, U.S.A.) using the carbodiimide coupling procedure. In brief, 3 mg of Met-E-Arg^6-Gly^7-Leu^8 and 36.6 mg of bovine thyroglobulin were dissolved in 2 ml of distilled water. To this solution was added 30 mg 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide HCl (Nakarai Chemicals, Co., Ltd., Kyoto Japan) in 1 ml of distilled water. The mixture was adjusted to pH 5.6 with 0.1 N HCl. After stirring for 13 hours at room temperature, the mixture was dialyzed against 4 l of distilled water at 4°C for 24 hours. Three mg of Met-E-Arg^6-Phe^7 was conjugated to 37.8 mg of bovine thyroglobulin in the same way.

Immunization

Conjugated Met-E-Arg^6-Gly^7-Leu^8 (100-200 μg) and Met-E-Arg^6-Phe^7 (100-200 μg) were emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan, U.S.A.) and used for immunizing New Zealand white rabbits by subcutaneous injections at multiple sites in the interscapulo-vertebral region. They were boostered every four weeks and bled 10-14 days after each booster injection.

Iodination

Met-E-Arg^6-Gly^7-Leu^8 and Met-E-Arg^6-Phe^7 were radioiodinated by the chloramine T method of Hunter and Greenwood (4). The labeled Met-E-Arg^6-Gly^7-Leu^8 and Met-E-Arg^6-Phe^7 were purified by applying the reaction mixture to a Sep-Pak C_{18} cartridge (Waters Associates, Inc., Milford, Minnesota, U.S.A.) filled with octadecylsilane-C_{18} (ODS-C_{18}) and eluting the labeled peptides with a solution of 80% methanol in 10 mM ammonium acetate, pH 4.2. The specific activity of ^{125}I-Met-E-Arg^6-Gly^7-Leu^8 and Met-E-Arg^6-Phe^7 ranged from 500 to 800 μCi/μg.

Radioimmunoassays (RIAs)

1) RIA for Met-E-Arg^6-Gly^7-Leu^8

The standard buffer for RIA was 0.05 M phosphate buffer (pH 7.4) containing 0.5% human serum albumin (Fraction V, MILES Laboratories, Inc., Elkhart, Indiana, U.S.A.), 0.1% Triton X-100 and 0.01% merthiolate and used to dissolve all reagents. The RIA incubation mixture consisted of 100 μl of standard Met-E-Arg^6-Gly^7-Leu^8 or sample, 100 μl of a final 1:4000 dilution of antiserum (NE2-206), 100 μl of ^{125}I-labeled Met-E-Arg^6-Gly^7-Leu^8 (approximately 2 x 10^4 cpm) and 200 μl of the standard buffer. The mixture was incubated for 24 hours at 4°C. Bound and free ligands were separated by adding 1.0 ml of a suspension of dextran-coated charcoal consisting of 200 mg Norit "Extra" (N.V. Norit-Vereenging, Holland) and 20 mg Dextran T-70 (Pharmacia Fine Chemicals, Uppsala, Sweden) in 100 ml of 0.05 M phosphate buffer, pH 7.4. Samples were routinely assayed in duplicate.

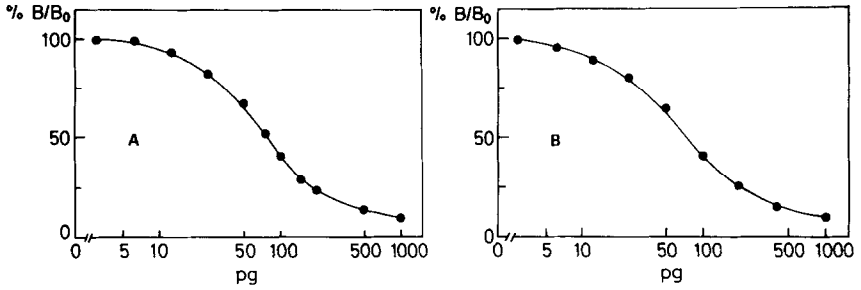

Fig. 1. Inhibition of binding of ^{125}I-labeled Met-E-Arg^6-Gly^7-Leu^8 to the Met-E-Arg^6-Gly^7-Leu^8 antiserum (NE2-206)(A) and of ^{125}I-labeled Met-E-Arg^6-Phe^7 to the Met-E-Arg^6-Phe^7 antiserum(AP3-311)(B) by unlabeled peptide. B/B_0 is expressed as percent.

2) RIA for Met-E-Arg^6-Phe^7

The RIA procedure was the same in all respects as that for Met-E-Arg^6-Gly^7-Leu^8, except that Met-E-Arg^6-Phe^7, the antiserum AP3-311 (final dilution 1:2000) and ^{125}I-labeled Met-E-Arg^6-Phe^7 (approximately 1 x 10^4 cpm) were substituted for the corresponding components.

3) RIAs for Met-E and Leu-E

Met-E and Leu-E were radioimmunoassayed as described previously (5).

Tissue and extraction procedure

Whole brains of male guinea pigs (350-400 g), Wister rats (250-300 g) and golden hamsters (110-120 g) were immediately obtained after decapitation. Extraction was performed by acidified methanol according to the procedure described previously (6). In brief, tissues were weighed and homogenized immediately in 10 volumes of acidified methanol consisting of equal parts of methanol and 0.1 N HCl, incubated for 10 min at 70°C and cooled on ice. The homogenate was centrifuged at 50,000 x g for 30 min at 4°C and the supernatant was stored at -20°C. The extracted samples were neutralized with 0.1 N NaOH immediately before RIA.

High performance liquid chromatography (HPLC)

The apparatus used for HPLC consisted of Shimadzu model LC-3A liquid chromatograph equipped with a SIL-IA injector and variable wave length UV detector SPD-2A (Shimadzu Corporation, Kyoto, Japan). Reversed phase HPLC was carried out on an Ultrasphere-ODS (Altex Scientific Inc., U.S.A.) column (4.6 x 150 mm). The extracts were directly applied to the column and eluted with 40% methanol in 10 mM ammonium acetate, pH 4.2, as a solvent. The flow rate was 1.0 ml/min and the fraction volume was 0.5 ml or 1 ml. The Met-E, Leu-E, Met-E-Arg^6-Gly^7-Leu^8 and Met-E-Arg^6-Phe^7 contents of each fraction were measured by respective RIAs. The retention times of synthetic Met-E, Leu-E, Met-E-Arg^6-Gly^7-Leu^8 and Met-E-Arg^6-Phe^7 monitored by ultraviolet absorbance or by RIAs were 6, 8, 16 and 21 min, respectively. Recoveries of Met-E, Leu-E, Met-E-Arg^6-Gly^7-Leu^8 and Met-E-Arg^6-Phe^7 standards applied on the column were 93%, 96%, 72% and 74%, respectively.

RESULTS

Typical standard curves with Met-E-Arg^6-Gly^7-Leu^8 and Met-E-Arg^6-Phe^7 of the RIAs are shown in Fig. 1A and 1B, respectively. Significant inhibition of the binding of ^{125}I-Met-E-Arg^6-Gly^7-Leu^8 and Met-E-Arg^6-Phe^7 to the corresponding antibodies were evident with as little as 15 pg of Met-E-Arg^6-Gly^7-

Table 1

Met-enkephalin-Arg^6-Gly^7-Leu^8-, Met-enkephalin-, Leu-enkephalin- and Met-enkephalin-Arg^6-Phe^7-like immunoreactivities in the whole brain of guinea pig, rat and golden hamster

	Guinea Pig (n=3)	Rat (n=6)	Golden Hamster (n=3)
Met-enkephalin-Arg^6-Gly^7-Leu^8-like immunoreactivity	20.5± 5.1	4.4±0.6	65.6± 2.8
Met-enkephalin-like immunoreactivity	113.4±12.9	33.8±3.4	139.6±11.3
Leu-enkephalin-like immunoreactivity	25.4± 1.1	12.2±0.8	35.0± 2.4
Met-enkephalin-Arg^6-Phe^7-like immunoreactivity	26.5± 3.0	5.0±0.5	80.9± 4.6

mean±SE ng/g wet weight

Leu^8·(NE2-202) showed no significant cross-reactivity with Met-E, Leu-E, Met-E-Arg^6-Phe^7, α-neo-endorphin, β-neo-endorphin, dynorphin, human β-endorphin and human ACTH (<0.01% on a molar basis). Met-E-Arg^6-Phe^7 antiserum (AP3-311) did not react significantly with Met-E, Leu-E, Met-E-Arg^6-Gly^7-Leu^8, α-neo-endorphin, β-neo-endorphin, dynorphin, human β-endorphin and human ACTH (<0.01% on a molar basis). The intra- and interassay coefficients of variation of Met-E-Arg^6-Gly^7-Leu^8 RIA were 3.4% and 6.6%, respectively, while those of Met-E-Arg^6-Phe^7 RIA were 5.4% and 7.2%, respectively. The dilution curves of extracts from the brains of guinea pigs, rats and golden hamsters were parallel with the standard curves of Met-E-Arg^6-Gly^7-Leu^8 and Met-E-Arg^6-Phe^7 in respective RIAs. The contents of Met-E-Arg^6-Gly^7-Leu^8-like immunoreactivity (LI), Met-E-LI, Leu-E-LI and Met-E-Arg^6-Phe^7-LI in guinea pig, rat and golden hamster brains are shown in Table 1. Met-E-Arg^6-Gly^7-Leu^8-LI was detected at concentrations comparable to those of Leu-E-LI and Met-E-Arg^6-Phe^7-LI in guinea pig, rat and golden hamster brains, though the content of Met-E-LI was the highest among the four opioid peptides.

The HPLC patterns of brain extracts of guinea pigs, rats and golden hamsters revealed one peak each of Met-E, Leu-E, Met-E-Arg^6-Gly^7-Leu^8 and Met-E-Arg^6-Phe^7 immunoreactivity eluting with the same retention times as those of synthetic peptides. The profile of each brain extract is shown in Fig. 2. The contents of Met-E, Leu-E, Met-E-Arg^6-Gly^7-Leu^8 and Met-E-Arg^6-

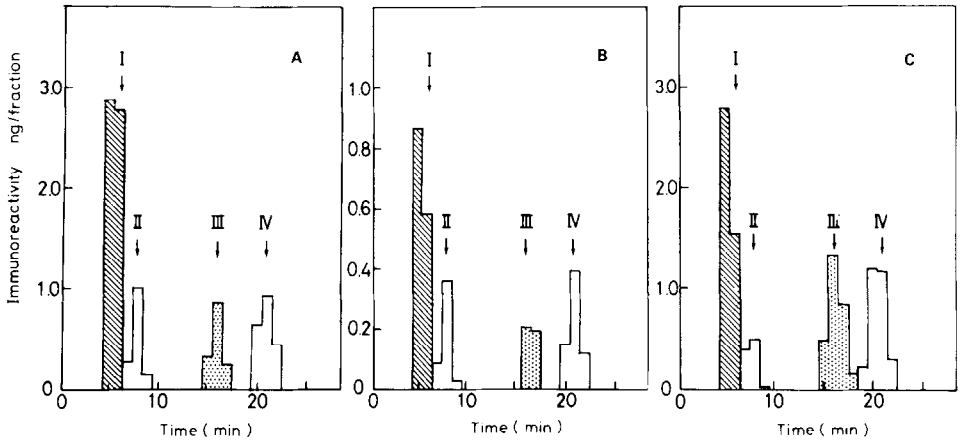

Fig. 2. High-performance liquid chromatographic profiles on an Ultrasphere-ODS column (4.6 x 150 mm) of brain extracts of guinea pig, rat and golden hamster. A: guinea pig, B: rat, C: golden hamster. Fractions of every 30 seconds were collected and assayed for Met-E, Leu-E, Met-E-Arg^6-Gly^7-Leu^8 and Met-E-Arg^6-Phe^7 immunoreactivities by respective RIAs. Arrows indicate the retention times of synthetic Met-E (I), Leu-E (II), Met-E-Arg^6-Gly^7-Leu^8 (III) and Met-E-Arg^6-Phe^7 (IV).

Phe^7 determined by HPLC coupled with the specific RIAs of each peptide are shown in Table 2. Considerable amounts of Met-E-Arg^6-Gly^7-Leu^8 were shown to exist with Met-E, Leu-E and Met-E-Arg^6-Phe^7 in guinea pig, rat and golden hamster brains. The Met-E content was the highest and the contents of Met-E-Arg^6-Gly^7-Leu^8 were comparable to those of Leu-E and Met-E-Arg^6-Phe^7.

DISCUSSION

Using synthetic Met-E-Arg^6-Gly^7-Leu^8 and Met-E-Arg^6-Phe^7, we were able to obtain sensitive and specific antisera and to set up RIAs for Met-E-Arg^6-Gly^7-Leu^8 and Met-E-Arg^6-Phe^7. The antiserum for Met-E-Arg^6-Gly^7-Leu^8 did

Table 2

Enkephalins and related peptides
in guinea pig, rat, and golden hamster brains

Compound	Retention time(min)	Guinea Pig	Rat	Golden Hamster
Met-enkephalin	6	139.2(79.9)	30.7(17.6)	140.6(80.7)
Leu-enkephalin	8	40.3(22.4)	9.90(5.52)	29.7(16.5)
Met-enkephalin-Arg^6-Gly^7-Leu^8	16	19.7(17.7)	2.50(2.24)	51.2(46.1)
Met-enkephalin-Arg^6-Phe^7	21	27.0(23.6)	3.61(3.17)	57.4(50.4)

pmole/g(ng/g)

not react with Met-E, Leu-E nor Met-E-Arg^6-Phe^7, and the Met-E-Arg^6-Phe^7 antiserum also did not react with Met-E, Leu-E nor Met-E-Arg^6-Gly^7-Leu^8. These results indicate that the two antisera recognize narrow C-terminal fragments. Therefore, the RIAs for Met-E-Arg^6-Gly^7-Leu^8 and Met-E-Arg^6-Phe^7 are useful tools for studying enkephalin-related peptides with close similarities in amino aicd sequence.

HPLC analysis coupled with RIAs for Met-E-Arg^6-Gly^7-Leu^8, Met-E, Leu-E and Met-E-Arg^6-Phe^7 showed the existence of Met-E-Arg^6-Gly^7-Leu^8 together with Met-E, Leu-E and Met-E-Arg^6-Phe^7 in the brain of guinea pig, rat and golden hamster. The contents of Met-E-Arg^6-Gly^7-Leu^8 in the brain of these animals are comparable to those of Leu-E and Met-E-Arg^6-Phe^7, though the content of Met-E is the highest among them. The peptide contents measured after HPLC were similar to those measured directly by RIAs.

These results are compatible with the recent studies on the nucleotide sequence of cDNA for bovine adrenal preproenkephalin, which predict the presence of four copies of Met-E and one copy each of Leu-E, Met-E-Arg^6-Phe^7 and Met-E-Arg^6-Gly^7-Leu^8 in this precursor molecule. Our results also suggest that the same biosynthetic precursor occurs in the central nervous system. Moreover, the precessing of preproenkephalin at paired basic amino acid residues is almost complete in the brain, because no considerable difference was found between the contents of these peptides determined after HPLC and those measured directly by RIAs in the brain. This is in contrast to our unpublished findings that the contents measured after HPLC were smaller in amount than those measured directly in human and bovine adrenal medullas.

Rossier et al (7) found that the content of Met-E-Arg^6-Phe^7 in the brain was in comparable range to that of Leu-E, using HPLC coupled with a N-terminal antiserum for enkephalins. Boarder et al(8) reported that a considerably larger amount of Met-E-Arg^6-Phe^7 was detected in brain tissues by specific RIA directed to sulfoxide Met-E-Arg^6-Phe^7. Our results show that the content

of Met-E-Arg^6-Phe^7 in the brain is comparable to that of Leu-E, in agreement with the results of Rossier et al, as well as to that of Met-E-Arg^6-Gly^7-Leu^8.

The existence of Met-E-Arg^6-Gly^7-Leu^8 in the brain of guinea pig, rat and golden hamster indicates a possible physiological significance of this peptide in the central nervous system. Further studies on the regional distribution of the octapeptide in the brain are ongoing in our laboratory.

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