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LEUMORPHIN IS A NOVEL ENDOGENOUS OPIOID PEPTIDE DERIVED FROM PREPROENKEPHALIN B

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Using synthetic leumorphin, we obtained antisera for leumorphin and set up two radioimmunoassays (RIAs) with different specificities. Gel exclusion chromatography coupled with the two RIAs showed the existence of a considerable amount of leumorphin-like peptide in water extracts from porcine neurointermediate pituitaries. Reverse phase high performance liquid chromatography revealed that leumorphin-like peptide in the water extracts was indistinguishable from synthetic leumorphin. These results along with potent opioid activity of leumorphin indicate that leumorphin is a novel endogenous opioid peptide derived from preproenkephalin B.

The primary structure of the common precursor of α-and β-neo-endorphin and dynorphin has recently been deduced from the nucleotide sequence of cloned DNA complementary to the porcine hypothalamic mRNA (1). This precursor, preproenkephalin B, contains a previously unknown peptide with 29 amino acids, which corresponds to amino acid residues 228-256 of preproenkephalin B and has leucine-enkephalin (leu-enkephalin) and rimorphin (dynorphin B) (2,3) at the N-terminal 1-5 and 1-13 portions, respectively. In addition, this peptide, named leumorphin, is flanked by paired basic amino acid residues, Lys-Arg, known to be a typical prohormone processing signal (4). More recently we have demonstrated that leumorphin has potent opioid activity (5,6,7) and acts as an agonist at the κ opioid receptor (5,6). Though rimorphin has been demonstrated

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Abbreviations used: leumorphin, Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr-Arg-Ser-Gln-Glu-Asp-Pro-Asn-Ala-Tyr-Tyr-Glu-Glu-Leu-Phe-Asp-Val; HPLC, high performance liquid chromatography; RIA, radioimmunoassay; EDTA, Ethylenediaminetetraacetic acid; LI, like immunoreactivity.

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to exist in association with α-neo-endorphin and dynorphin in bovine, porcine and rat posterior pituitaries and neural tissues (2,8) and the human hypothalamus (9), endogenous existence of leumorphin has not yet been proved.

In order to elucidate whether or not leumorphin is a novel endogenous opioid peptide, we have studied extracts from porcine neurointermediate pituitaries using gel exclusion chromatography and high performance liquid chromatography (HPLC) coupled with radioimmunoassays (RIAs) for leumorphin with different specificities.

MATERIALS AND METHODS

Peptides

Leumorphin and leumorphin(15-29) were synthesized by a solid phase method (7). Rimorphin and 4000-dalton dynorphin were donated by Peninsula laboratories.

Conjugation of leumorphin

Leumorphin was conjugated to bovine thyroglobulin(Sigma) using the carbodiimide coupling procedure (10).

Immunization

Conjugated leumorphin(100-200 μg) was emulsified with an equal volume of Freund's complete adjuvant (Difco) and used for immunizing New Zealand white rabbits as previously described (11).

Iodination

Leumorphin and rimorphin were radioiodinated by the chloramine T method (11). The specific activities of ^{125}I-leumorphin and ^{125}I-rimorphin ranged from 300 to 500 μCi/μg.

Radioimmunoassays (RIAs)

1) RIA for leumorphin and rimorphin (HLM-II-114)

The standard buffer for RIA was 0.05 M phosphate buffer (pH 7.4) containing 0.1% human serum albumin (Fraction V, MILES), 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 leumorphin or sample, 100 μl of final 1:2000 dilution of antiserum (HLM-II-114), 100 μl of ^{125}I-rimorphin (approximately 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 0.5 ml of a suspension of dextran-coated charcoal consisting of 200 mg of Norit SX Plus (Norit AG) and 20 mg of Dextran T-70 (Pharmacia) in 100 ml of 0.05 M phosphate buffer (pH 7.4) containing 0.01% merthiolate.

2) RIA for leumorphin (PLM-I-1208)

The standard buffer was the same as that ot the RIA of HLM-II-114. The RIA incubation mixture consisted of 100 μl of standard leumorphin or sample, 100 μl of a final 1:1000 dilution of antiserum(PLM-I-1208), 100 μl of ^{125}I-leumorphin(approximately 10^4 cpm) and 200 μl of the standard buffer. The mixture was incubated for 48 hours at 4°C. Bound and free ligands were separated by adding 0.5 ml of a suspension of dextran-coated charcoal consisting of 350 mg of Norit SX Plus and 35 mg of Dextran T-70 in 100 ml of 0.05 M phosphate buffer (pH 7.4) containing 0.01% merthiolate.

Tissue and extraction procedure

Fresh porcine pituitaries were obtained from a slaughterhouse and neurointermediate lobes were immediately dissected out, frozen in liquid nitrogen and stored at -30°C until extraction. Porcine neurointermediate pituitaries were boiled in 5 volumes of distilled water containing phenylmethylsulfonylfluoride (20 μg/ml), pepstatin A (1 μg/ml), aprotinin (2000 KIU/ml), leupeptin (6 μg/ml), bacitracin (200 μg/ml), N-ethylmaleimide (20 μg/ml) and EDTA (5 mM)

for 10 minutes and homogenized with Polytron homogenizer. The homogenate was centrifuged at 50,000 x g for 30 minutes at 4°C and the supernatant was stored at -30°C and used as water extracts. Subsequently the pellet was re-extracted in 5 volumes of 1 M acetic acid. After centrifugation, the supernatant was also stored at -30°C and used as acid extracts.

Gel exclusion chromatography

The sample reconstituted in 4 M guanidine HCl was applied on a 0.7 x 50 cm column of Bio-Gel P-6 equilibrated with 4 M guanidine HCl at 4°C. The flow rate was 1 ml per hour and the fraction volume was 0.63 ml. The column was calibrated with blue dextran(void volume), leumorphin, rimorphin and ^{125}I (salt peak).

High performance liquid chromatography (HPLC)

Reverse phase HPLC was carried out on a TSK-GEL ODS-120T, 5μ(TOYO SODA MANUFACTURING CO.LTD. Japan)column(4.6 x 250 mm). The water extracts were applied to the column and eluted with 30% CH_3CN in 5 mM trifluoroacetic acid containing 50 mM ammonium sulphate isocratically. The flow rate was 1 ml per minute and the fraction volume was 0.5 ml. The retention times of synthetic leumorphin, rimorphin and 4000-dalton dynorphin were monitored by ultraviolet absorption or the RIAs.

RESULTS

RIAs

Typical standard curves of leumorphin in the two RIAs are shown in Fig.1. Significant inhibition of the binding of ^{125}I-rimorphin to the antiserum, HLM-II-114, was evident as little as 10 pg of leumorphin and 2 pg of rimorphin. The RIA recognized rimorphin 3.2 times more than leumorphin on a molar basis and had a cross-reactivity of 10% with 4000-dalton dynorphin, but no significant cross-reactivity with leu-enkephalin, leumorphin(15-29), dynorphin(1-17) and (1-8), α-and β-neo-endorphin, met-enkephalin, met-enkephalin-Arg^6-Phe^7, met-enkephalin-Arg^6-Gly^7-Leu^8, β-endorphin and ACTH (<0.01% on a molar basis). The other RIA for leumorphin using the antiserum, PLM-I-1208, could detect 10 pg of leumorphin and reacted weakly with leumorphin(15-29)(0.2% on a molar basis). This RIA did not cross-react significantly with rimorphin, 4000-dalton

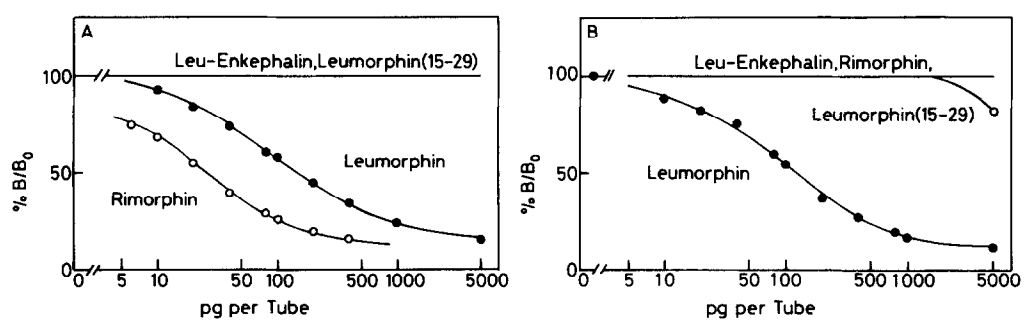

Fig. 1. Specificities of leumorphin RIAs using the antiserum HLM-II-114 (A) and the antiserum PLM-I-1208 (B).

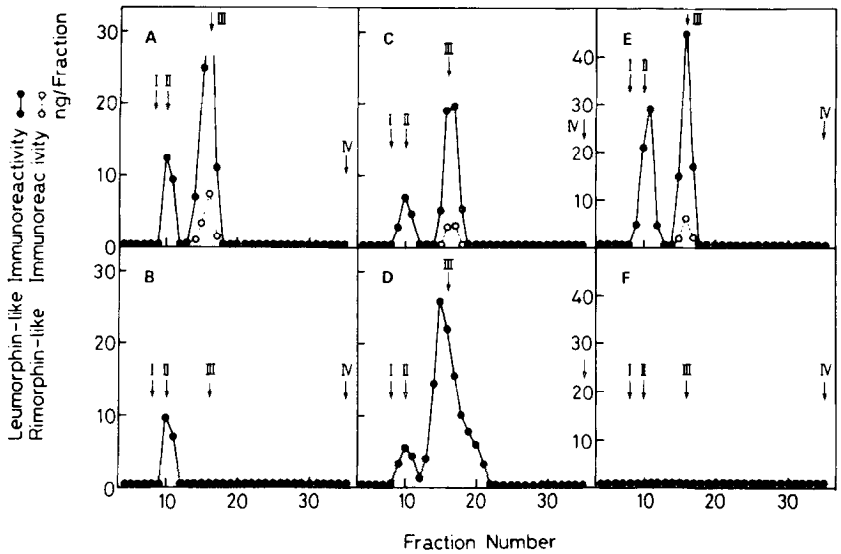

Fig. 2. Gel filtration profiles of standard leumorphin and rimorphin (A,B), water extracts (C,D) and acid extracts (E,F) from porcine neurointermediate pituitaries. A,C and E were assayed by the RIA of HLM-II-114. B,D and F were measured by the RIA of PLM-I-1208. Arrows indicate the elution positions of void volume (I), leumorphin (II), rimorphin (III), and salt (IV).

dynorphin, leu-enkephalin and other endogenous opioid peptides mentioned above (<0.01% on a molar basis).

Gel exclusion chromatography

Gel chromatographic profiles of standard leumorphin and rimorphin, the water extracts and the acid extracts from porcine neurointermediate pituitaries are depicted in Fig.2. As seen in Fig. 2C, gel chromatography of the water extracts coupled with the RIA of HLM-II-114 showed two peaks of leumorphin-like immunoreactivity(leumorphin-LI) eluting at the corresponding positions of leumorphin and rimorphin, respectively, which is quite similar to an elution profile of standard leumorphin and rimorphin shown in Fig.2A. The determination with the other RIA of PLM-I-1208 revealed two components of leumorphin-LI emerging at the position of leumorphin and in the relatively broad range near the elution position of rimorphin, respectively, as shown in Fig.2D. The amount of leumorphin-LI eluting at the position of synthetic leumorphin determined by the RIA of HLM-II-114 was about the same as that measured by the RIA of PLM-I-1208 The content of leumorphin-like peptide which was detected by gel filtration coupled with these two RIAs was 154 ng per g tissue. The acid extracts showed

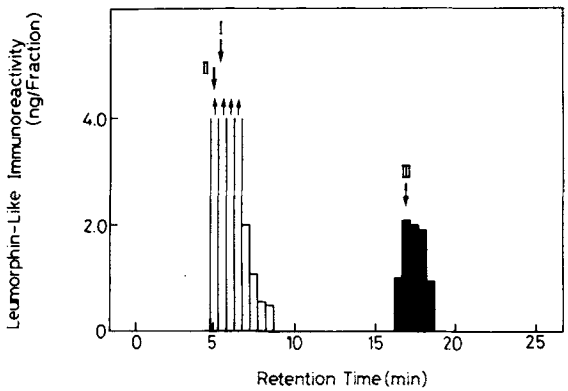

Fig. 3. A HPLC profile of water extracts from porcine neurointermediate pituitaries on a TSK-GEL ODS-120T column. The leumorphin content of each fraction was measured by the RIA of HLM-II-114. Arrows indicate the retention times of synthetic rimorphin (II), 4000-dalton dynorphin (I) and leumorphin (III).

two peaks of leumorphin-LI when measured by the RIA of HLM-II-114 as seen in Fig.2E, but no leumorphin-LI could be detected in the acid extracts by the RIA of PLM-I-1208 as shown in Fig.2F. The total content of rimorphin in the water and acid extracts was 503 ng per g tissue.

HPLC

HPLC analysis of the water extracts from porcine neurointermediate pituitaries revealed a peak of leumorphin-like peptide eluting at the same retention time with standard leumorphin as exhibited in Fig.3.

DISCUSSION

Using synthetic leumorphin, we raised antisera for leumorphin and set up two RIAs which recognized different portions of leumorphin. One RIA recognized rimorphin or N-terminal 1-13 of leumorphin, whereas the other RIA did not react with rimorphin. This result indicates that leumorphin may contain at least two highly antigenic sites in the molecule. The two RIAs for leumorphin with different specificities established in the present study are useful tools for searching for leumorphin in various tissues.

α-and β-neo-endorphin, dynorphin and rimorphin are all basic peptides, whereas leumorphin is an acidic peptide containing four basic amino acids in its N-terminal fragment and five acidic amino acids in its C-terminal fragment. Therefore, taking acidic nature of leumorphin into consideration, we chose an

extraction procedure of boiling water and a subsequent acetic acid extraction. In addition, to minimize artificial proteolysis during the extraction, we used proteolytic enzyme inhibitors.

The gel chromatographic profile of the water extracts was clearly different from that of the acid extracts. A considerable amount of leumorphin-like peptide which could be detected by the both RIAs with different specificities was obtained with the water extraction, but leumorphin-like peptide could not be observed in the acid extracts. Most of rimorphin was obtained with the acid extraction. These findings are consistent with the physicochemical natures of leumorphin and rimorphin. Similar results have been reported in extraction of multiple forms of cholecystokinin(12). The content of rimorphin in the porcine neurointermediate pituitary in the present study is in agreement with the result of Kilpatrick et al (2) and approximately half of that reported by Cone et al (8). Another interesting finding in this study is occurrence of the peak of leumorphin-LI in the acid extracts with a similar molecular weight to that of leumorphin detectable only by the RIA of HLM-II-114. The nature of this peptide is not clear at present. It is possible to speculate that it may contain N-terminally extended rimorphin or a 4000-dalton dynorphin(3). The exact nature of this fraction must await further clarification.

In addition to the gel chromatographic findings, HPLC analysis has clearly shown that leumorphin-like peptide in the water extracts is indistinguishable from synthetic leumorphin.

These results along with our recent observations on potent opioid activity of leumorphin(5,6,7) indicate that leumorphin is a novel endogenous opioid peptide derived from preproenkephalin B.

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