Vol. 123, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1984 Pages 148-155

LEUMORPHIN IS A NOVEL ENDOGENOUS OPIOID PEPTIDE IN MAN

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Received July 16, 1984

Porcine leumorphin, a putative opioid peptide corresponding to amino acid residues 228-256 of preproenkephalin B has been demonstrated to exist in the porcine neurointermediate pituitary. A recent study on the sequence analysis of genomic DNA of human preproenkephalin B has shown that human leumorphin differs in 3 amino acid residues from porcine leumorphin. In order to clarify whether leumorphin is an endogenous opioid peptide in man, we have studied its existence in the human brain using a radioimmunoassay and its opioid activity by a bioassay with the guinea-pig ileum myenteric plexus-longitudinal muscle preparation. High performance gel permeation chromatography and reverse-phase high performance liquid chromatography coupled with the radioimmunoassay for leumorphin have revealed that human leumorphin exists in water extracts of the human striatum. In the guinea-pig ileum assay, synthetic human leumorphin exhibited potent opioid activity, with the concentration of 3nM to give 50 per cent inhibition. These results indicate that leumorphin is a novel endogenous opioid peptide in man.

The primary structure of the common precursor of neo-endorphin and dynorphin was deduced from the nucleotide sequence of cloned DNA complementary to the porcine hypothalamic messenger RNA. This precursor, preproenkephalin B, contains a third leucine enkephalin sequence with a carboxyl-terminal extension of 24 amino acids at its carboxyl-terminus (1,2). Since this 29 amino acids sequence is preceded by paired basic amino acid residues, lysine-arginine (Lys-Arg)[1], known to be a typical prohormone processing

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[1] Abbreviations used in this paper: Lys-Arg, lysine-arginine; HP-GPC, high performance gel permeation chromatography; RP-HPLC, reverse-phase high performance liquid chromatography; RIA, radioimmunoassay; TFA, trifluoroacetic acid; IC_{50}, the concentration giving 50 percent inhibition; -LI, -like immunoreactivity.

0006-291X/84 $1.50

signal (3), it was assumed to be a novel opioid peptide and named leumorphin. Recently, we have reported that porcine leumorphin has potent opioid activity (2,4,5), acts as an agonist at the κ type opioid receptor (2,4) and exists in porcine neurointermediate pituitaries (6). Thus, leumorphin, a putative opioid peptide predicted from the nucleotide sequence of cloned cDNA for proenkephalin B has been demonstrated to be a new endogenous opioid peptide. More recently, the gene sequence analysis of human preproenkephalin B has revealed that human leumorphin differs in three amino acid residues from porcine leumorphin (7). Although we have demonstrated that rimorphin (8,9), a peptide corresponding to the N-terminal 13 amino acid residues of leumorphin, exists together with α-neo-endorphin and dynorphin in the human brain (10), the existence of leumorphin in man has not yet been proved. In order to elucidate whether or not leumorphin is a novel endogenous opioid peptide in man, we have studied extracts from a human striatum using high performance gel permeation chromatography (HP-GPC) and reverse-phase high performance liquid chromatography (RP-HPLC) coupled with a radioimmunoassay (RIA) for human leumorphin. We have also studied the biological activity of synthetic human leumorphin with the guinea-pig ileum myenteric plexus-longitudinal muscle preparation.

METHODS

Compounds

Human leumorphin and human leumorphin (15-29) were synthesized by a solid phase method. Rimorphin, 4000 dalton dynorphin and dynorphin (1-24) were kindly donated by Peninsula laboratories (San Carlos, Calif.). Dynorphin (1-17) and dynorphin (1-8) were provided by Drs. M. Fujino and M. Wakimasu (Central Research Division, Takeda, Chemical Industries, Ltd., Osaka, Japan). α-and β-neo-endorphin were gifts of Dr. H. Matsuo (Miyazaki Medical College, Miyazaki, Japan). Methionine- and leucine-enkephalin and human β-endorphin were supplied by Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan and Professor C.H. Li (University of California, San Francisco), respectively. Naloxone hydrochloride was provided by Endo Laboratories Inc, Garden City, N.Y..

RIA

RIA for human leumorphin was performed according to the method described previously (6). An antiserum for human leumorphin (HLM-II-802) was raised in a New Zealand white rabbit by injecting synthetic human leumorphin conjugated to bovine thyroglobulin (Sigma Chemical Co., St Louis, MO.) as an antigen. Significant inhibition of the binding of ^{125}I-rimorphin to the antiserum, HLM-II-802, was evident by the addition of as little as 5pg of human leumorphin and 1pg of rimorphin. This RIA recognized rimorphin 2.3 times more than human leumorphin on a molar basis and had a cross-reactivity of 10% with 4000 dalton dynorphin, but no significant cross-reactivity with leucine-enkephalin, human leumorphin (15-29), dynorphin (1-24), dynorphin (1-17),

dynorphin (1-8), α- and β-neo-endorphin, methionine-enkephalin and human β-endorphin (< 0.01%).

Sample and tissue extraction

A human brain was obtained at autopsy within 3 hours after death from an adult patient without neurological disorders. The striatum was carefully dissected out and stored at -70°C until extraction. Tissue extraction was performed as previously described (6). In brief, the striatum was weighed and boiled for 10 minutes at 96°C and homogenized in 10 volumes of distilled water containing phenylmethylsulfonylfluoride (Boehringer Mannheim Gmb, West Germany) at 20 μg/ml, pepstatin A (Protein Research Foundation, Osaka, Japan) at 1 μg/ml, aprotinin (Ookura Pharmaceutical Co., Ltd., Kyoto, Japan) at 2000 KIU/ml, leupeptin (Protein Research Foundation, Osaka, Japan) at 6 μg/ml, bacitracin (Sigma Chemical Co., St Louis, MO.) at 200 μg/ml, N-ethylmaleimide (Nakarai Chemicals, Co., Ltd., Kyoto, Japan) at 20 μg/ml and EDTA (Nakarai Chemicals, Co., Ltd., Kyoto, Japan) at 5 mM. The homogenate was centrifuged at 50,000 x g for 30 minutes at 4°C and the supernatant was stored as a water extract. Subsequently, the pellet was re-extracted in 10 volumes of 1M acetic acid and used as an acid extract.

Condensation of tissue extracts

Before HP-GPC and RP-HPLC analyses, the water and acid extracts of the striatum were passed through C_{18} Sep-Pak cartridges (Waters Associates, Inc., Milford, Mass.) preequilibrated with 5mM trifluoroacetic acid (TFA). The cartridges were first washed with 20 ml of 5 mM TFA and the peptides were then eluted with 2 ml of 50% acetonitrile in 5 mM TFA. Recoveries of synthetic human leumorphin and rimorphin from the cartridges were both more than 90%.

HP-GPC

HP-GPC was carried out on a TSK-GEL G2500 PW (Toyo Soda Manufacturing Co., Ltd., Tokyo, Japan) column (7.5 x 600 mm) and eluted with 10 mM TFA containing 0.2 M sodium chloride and 30% acetonitrile, as a solvent. The flow rate was 0.3 ml/min and the fraction volume was 0.12 ml. The elution positions of peptides were monitored by ultraviolet absorbance at 210 nm or by RIA.

RP-HPLC

RP-HPLC was performed on a TSK-GEL 120T octadecylsilane column (5 particle size: 4.6 x 75 mm) (Toyo Soda Manufacturing Co., Ltd., Tokyo, Japan). Peptides were eluted from the column isocratically with 23% acetonitrile in 5mM TFA. The flow rate was 1 ml/min and the fraction volume was 0.5 ml. The retention times of synthetic rimorphin, 4000 dalton dynorphin and human leumorphin were 4.0, 10.0 and 14.5 min, respectively.

Bioassay

Male Hartley guinea-pigs weighing 300-500 g were used. Opioid activity of synthetic human leumorphin was evaluated using the guinea-pig ileum myenteric plexus-longitudinal muscle bioassay as described (2). The concentration of human leumorphin required to inhibit the muscle twitch by 50% (IC_{50}) was determined by testing at three or more concentrations giving 20-80% inhibition and then interpolating IC_{50} by the log-linear regression analysis.

RESULTS

Identification of human leumorphin

As seen in Fig. 1a, HP-GPC of the water extract of a human striatum showed three peaks of leumorphin-like immunoreactivity (-LI) eluting at the positions corresponding to those of 4000 dalton dynorphin (peak A), human leumorphin (peak B) and rimorphin (peak C), respectively. RP-HPLC analysis of the water extract (Fig. 1b) revealed apparently two peaks of leumorphin-LI with

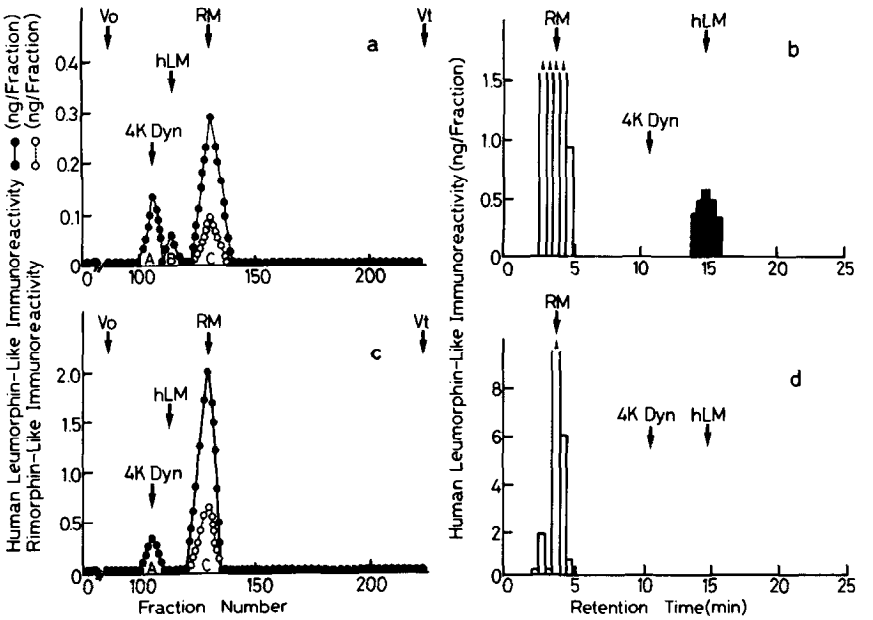


FIGURE 1. High performance gel permeation chromatographic (HP-GPC) and reverse-phase high performance liquid chromatographic (RP-HPLC) profiles of water and acid extracts of a human striatum on a TSK-GEL G2500 PW column (7.5 x 600 mm) and a TSK-GEL ODS-120T column (4.6 x 75 mm), respectively. (a) a HP-GPC profile of a water extract from the human striatum, (b) a RP-HPLC profile of the water extract, (c) a HP-GPC profile of an acid extract, (d) a RP-HPLC profile of the acid extract. Fraction obtained from the columns were assayed with leumorphin RIA. Arrows indicate the elution positions of void volume (Vo), synthetic 4000 dalton dynorphin (4K Dyn), human leumorphin (hLM), rimorphin (RM) and salt (Vt).

the same retention times as those of synthetic human leumorphin and rimorphin, respectively. No detectable amounts of leumorphin-LI could be seen at the elution position of synthetic 4000 dalton dynorphin (Fig. 1b).

Leumorphin-LI eluting with the retention time identical to human leumorphin in RP-HPLC was rechromatographed by HP-GPC on a TSK-GEL G2500PW column. This immunoreactivity was eluted at the position corresponding to that of synthetic human leumorphin shown as peak B in Fig. 1a. HP-GPC of leumorphin-LI emerging at the position of rimorphin in RP-HPLC showed that this peak consisted of two components, rimorphin, peak C, and another leumorphin-LI, peak A, as shown in Fig. 1a.

On the other hand, HP-GPC of the acid extract revealed only two peaks of leumorphin-LI (peak A and C) as shown in Fig. 1c. RP-HPLC analysis of the acid extract showed no detectable amounts of leumorphin and 4000 dalton

dynorphin, but there were two peaks of leumorphin-LI, one eluting at the position of synthetic rimorphin and another small peak emerging somewhat faster than rimorphin (Fig. 1d). The ratio of these two peaks were almost equal to that of peak A and peak C on HP-GPC.

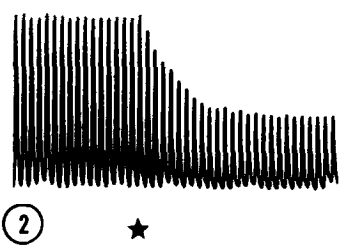
The contents of leumorphin and rimorphin calculated as sums of their contents in water and acid extracts of the human striatum determined after HP-GPC and RP-HPLC were 4.8 ng/g and 15.8 ng/g wet weight, respectively.

Opioid activity of human leumorphin

Electrical stimulation of the intramural nerves of the guinea-pig ileum caused twitch-like contraction. The additions of isolated and synthetic human leumorphin significantly depressed the electrically evoked contraction of the muscle strip (Fig. 2). These effects in this tissue were antagonized effectively by naloxone, a specific opioid receptor antagonist. An IC_{50} value of synthetic human leumorphin was 3.05 $\pm$ 0.62 nM (mean $\pm$ SE of 6 experiments).

DISCUSSION

The diagram of the structure of the porcine preproenkephalin B, a common precursor of neo-endorphin, dynorphin and leumorphin is shown in Fig. 3. The position of paired and single basic amino acid residues, typical processing



(2)

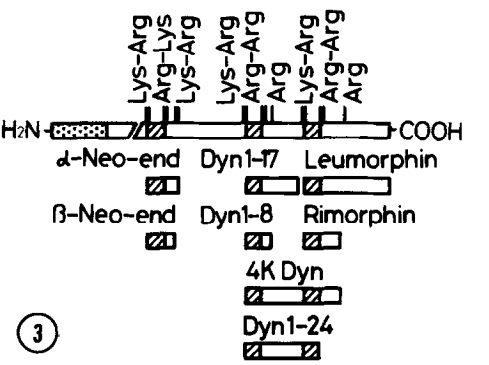


FIGURE 2. Effects of synthetic human leumorphin on the electrically evoked contraction of the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum. Human leumorphin at a final concentration of 3 nM was added at the point shown by an asterisk.

FIGURE 3. Schematic representation of porcine preproenkephalin B. Leucine-enkephalin sequences and the putative signal peptide sequence are expressed by hatched and dotted boxes, respectively. The position of a single and paired basic amino acid residues, possible cleavage sites are also shown. Characterized peptides derived from porcine preproenkephalin B are indicated below.

signals (3), and proenkephalin B derived opioid peptides characterized up to now are also presented in Fig. 3. Recently, the primary structure of human preproenkephalin B deduced from the sequence of genomic DNA has revealed that neo-endorphin, dynorphin and rimorphin (dynorphin B) (8,9) are identical to their porcine counterparts in amino acid sequence, whereas the carboxyl-terminal sequence of human leumorphin differs in three amino acid residues from porcine leumorphin(7).

Using HP-GPC and RP-HPLC coupled with a RIA for human leumorphin, we have demonstrated the presence of leumorphin in the striatum of human brain. It is of particular interest that human leumorphin was extracted in the boiling water more effectively than in the 1M acetic acid. The observation that human leumorphin was preferentially extracted under neutral condition and rimorphin was extracted under acid condition is consistent with the our previous finding with porcine neurointermediate pituitaries (6). These results can be explained by the acidic nature of human and porcine leumorphin in contrast to the basic nature of neo-endorphin, dynorphin and rimorphin. A similar finding has been reported that cholecystokinin 8 is well extracted by boiling tissue at neutral pH, but is virtually insoluble at acid pH, which probably explains why this molecular form was not detected in the original isolation of cholecystokinin 33 (11).

Another interesting finding in this study is the presence of a leumorphin-LI whose elution position on HP-GPC corresponded to that of 4000 dalton dynorphin. However, this immunoreactive material migrated faster on RP-HPLC than did 4000 dalton dynorphin and human leumorphin. Our observation that the leumorphin-LI of peak A depicted in Fig. 1 a,c was almost equal in molecular size to those of 4000 dalton dynorphin and human leumorphin and had less hydrophobic nature on RP-HPLC suggests the possible involvement of chemical modifications of 4000 dalton dynorphin and/or human leumorphin during the posttranslational processing of human preproenkephalin B. It should be noted that O-sulfated leucine-enkephalin are present in rat, sheep, mouse and guinea-pig striatum (12). Moreover, the sequences, -serine^{15}-

glutamine^{16}-glutamic acid^{17}- and -serine^{23}-glycine^{24}-glutamic acid ^{25} - in human leumorphin are potential sites for phosphorylation (13). Further characterization of this unknown peptide is on going in our laboratory.

The presence of the tyrosine^{1}-glycine^{2}-glycine^{3}-phenylalanine^{4}- leucine^{5}-arginine^{6}-arginine^{7}-sequence at the amino terminus of human leumorphin, as in porcine leumorphin and dynorphin, suggested that it too would exhibit potent opioid activity in the guinea-pig ileum preparation. Synthetic human leumorphin was indeed found to be 150 times more active than free leucine-enkephalin, with an IC_{50} of 3nM. This value is almost equal to those of porcine leumorphin and rimorphin in our previous report (4). In addition, our unpublished observation shows that human leumorphin acts as an agonist at the κ type opioid receptor like porcine leumorphin. Since κ binding sites are known to be predominant among multiple opioid binding sites in the human brain (14), the endogenous existence of human leumorphin and its potent opioid activity demonstrated in this study strongly suggest that human leumorphin plays an important physiological role in the human central nervous system.

ACKNOWLEDGEMENTS

We are indebted to Dr. Jaw-Kang Chang, Peninsula Laboratories, Inc. for the generous supply of synthetic peptides; to Dr. Y. Kato and Dr. H. Moriyama, Central Research Laboratory, Toyo Soda Manufacturing Co., Ltd., for the generous supply of TSK-GEL columns and for valuable technical advice; to Dr. Y. Ikeda, S. Oki and I. Tanaka for valuable discussion. We also thank Miss K. Yada and Miss Y. Nakanishi for the secretarial assistance. This work was supported in part by special coordination funds for promoting science and technology, by a Grant-in-Aid for cancer Research (56-6) from the Japanese Ministry of Health and Welfare and by a research grant from the Naito Foundation.

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