Vol. 202, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1994 Pages 410-415

ISOLATION OF ENDOGENOUS HEMORPHIN-RELATED HEMOGLOBIN FRAGMENTS FROM BOVINE BRAIN

Andrey A. Karelin, Marina M. Philippova, Elena V. Karelina and Vadim T. Ivanov

Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia

Received May 25, 1994

Summary: Six short-chain peptides were isolated from an acidic extract of bovine brain in the course of total peptide screening. Their primary structures determined by Edman degradation were LVVYP, LVVYPWT, LVVYPWTQ, LVVYPWTQRF, VVYPWTQ and VVYPWTQRF, which respectively corresponded to the fragments 31-35, 31-37,31- 38, 31-40, 32-38 and 32-40 of bovine hemoglobin β-chain. All these peptides contained sequences of opioid peptides - hemorphins. For two of these peptides, viz. 32-38 and 31-40, isolated from other sources, an opioid activity was demonstrated formerly [1,2].

A number of peptides isolated from enzymatically treated hemoglobin exhibit an opioid activity [3]. The amino acid sequences of these peptides (34-37 and 34-38 residues) were identified in the structure of the hemoglobin β-chain(h.l. and hereinafter the numbering of amino acid residues corresponds to the bovine hemoglobin sequence). These peptides were named hemorphins. At first other fragments of this protein (31-40 and 32-40) were isolated as an accessory product of purification from the porcine hypothalamus, but biological activity of these peptides was not investigated [4]. Then the first of these peptides (LVV-hemorphin-7) was isolated from cerebrospinal fluid of patients with cerebrovascular bleedings [2]. The opioid-like activity of this peptide was determined. Homologous hemorphin-containing peptide fragments were isolated from different biological sources, such as human pituitary gland [5], porcine bone marrow [6] and bovine hypothalamus [7].

During the total screening of peptide material from acidic extract of bovine brain six homologous peptides, containing hemorphin sequences, were isolated, three of them (32-38, 31-40 and 32-40) being earlier isolated from other sources. The content of all six peptides in the bovine brain tissue was determined [8].

Materials and methods

Tissue extraction. Cortex and subcortex were cut from bovine brain (2.5 kg, 6 animals) frozen in liquid nitrogen and separately extracted. Subcortex (1.2 kg) was extracted with

0006-291X/94 $5.00

10 % acetic acid in knife homogenizer (12000 rev/min, 3 min). The mixture was centrifuged in a K70 centrifuge (Janetsky, Germany) at 8000 rev/min for 20 min. The supernatant was collected and lyophilised.

Size-exclusion chromatography. Size-exclusion chromatography was performed on a Sephadex G-25 superfine (Pharmacia, Sweden) column (2.5 x 90 cm) eluted with 0.1 M acetic acid at 60 ml/h flow rate, the fractions were collected as shown in Fig. 1.

High-performance liquid chromatography (HPLC). For reversed- phase separation a Nucleosil 7C8 (Macherey-Nagel, Germany) column (4.6 x 250 mm) was used. The sample (5 mg) was eluted with a linear gradient (0-60 %) of acetonitrile in 0.1 % trifluoracetic acid (TFA). The fractions containing main peaks were lyophilised and rechromatographed with a linear gradient (40-60%) of acetonitrile in 0.1% TFA as shown in Figures 3 a-d.

Peptide sequencing. Sequencing of the isolated peptides was performed with the Applied Biosystem 477 A gas-phase sequencer (Foster City, USA).

Results

The total bovine brain was frozen in liquid nitrogen and the cortex was separated from subcortical structures of frozen brain. Subcortical structures were extracted with 10 % acetic acid, centrifuged and the supernatant was lyophilised. We obtained one gram of extract from 20 g of crude material. Then the extract was fractionated on Sephadex G-25 sf column. The resultant highly reproducible elution profile is shown in Fig. 1. Peptides 5-20 amino acid residues in length comprised the fraction B, while the fraction A contained longer peptides (up to 40-45 amino acid residues). 800 mg of extract afforded 60 mg of fraction B in one chromatographic run.

The peptide material was analyzed by RP-HPLC on Nucleosil 7C8 column (Fig. 2). The fractions containing main peaks were rechromatographed (Fig. 3a-d) and sequenced in the gas-phase sequencer. The obtained results were compared with the sequences available in the protein data banks. Five short peptides containing hemorphin sequence

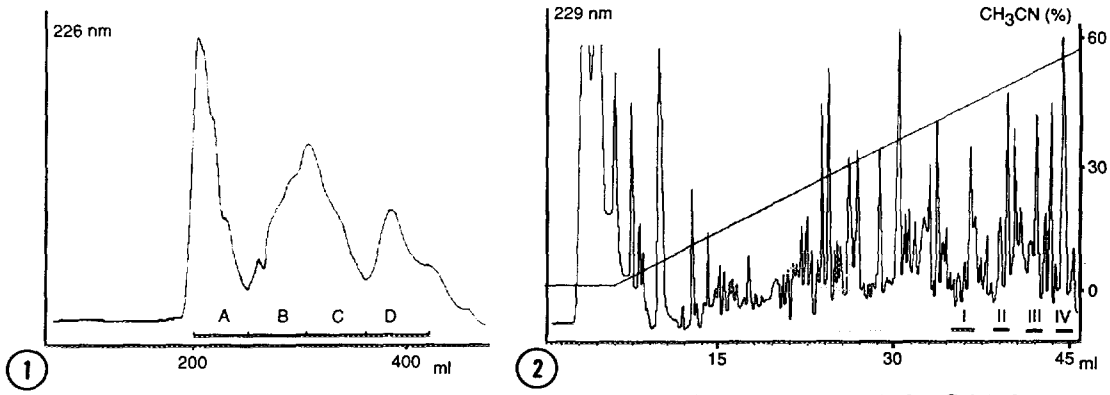

Fig. 1. Size-exclusion chromatography of bovine brain acidic extract on Sephadex G-25 sf column. Collected fractions are marked.

Fig. 2. Reversed-phase separation of fraction B (Fig. 1) on Nucleosil 7C8 column. Analyzed fractions are marked.

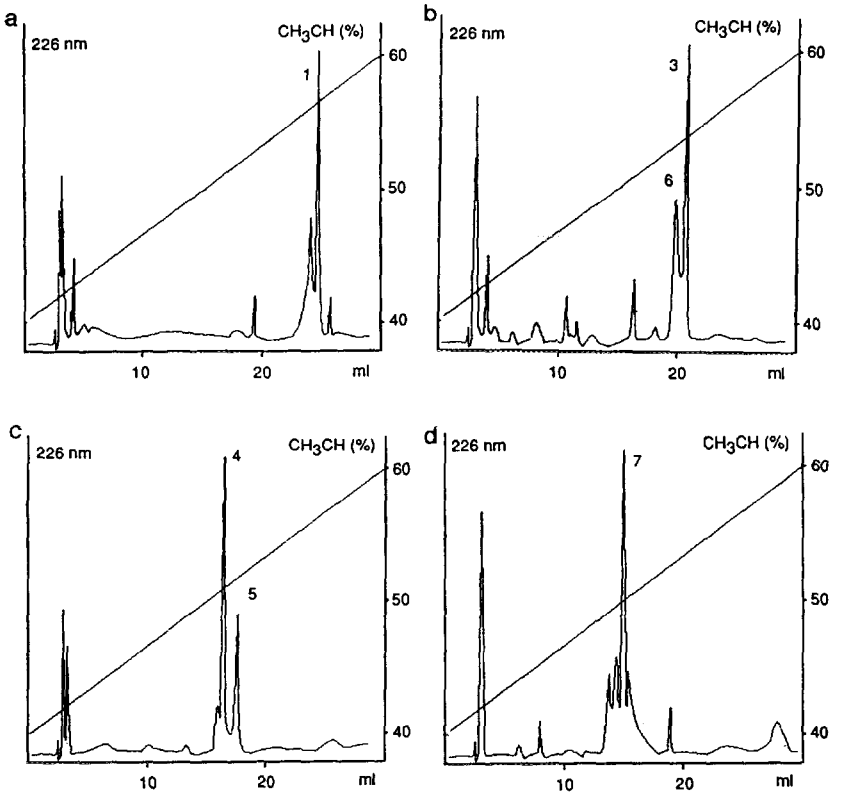

Fig. 3. Isolation of homogeneous peptides for sequencing on standart RP-HPLC conditions. Peaks of identified peptides are numerated. Peptide numbering corresponds to Table 1; a. Fraction I; b. Fraction II; c. Fraction III; d. Fraction IV.

and a peptide containing Tyr- residue from hemorphin active site 31-35 were found. All of these peptides, which presented in fraction B, are marked (Fig. 2).

Levels of peptide content in all analyzed peaks were established by PTH-derivatives output during the sequencing. The results are summurized in Table 1.

Discussion

Peptide fragments (Table 2) exhibiting opioid-like activity have been obtained as a result of enzymatic treatment of functional proteins, such as β-caseins [9], cytochrom B [10], hemoglobin [11]. All these sequences are highly homologous. Moreover, other peptide fragments of the α-chain of bovine hemoglobin (kyotorphin (140-141) and neo-kyotorphin (137-141)), exhibited opioid-like activity, were identified *in vivo* [12,13]. The presence of hemoglobin fragments, exhibited the opioid-like activity in the organisms of different mammals species, allows to conclude the existence of peptidergic regulation of opioid system, which differs from regulation by "classical" opioid peptides (such as enkephalins, endorphins or dynorphins).

Table 1. Endogenous hemorphin-containing peptides from the β-chain of hemoglobin

Code	Sequence	Position	Content nmol/g tissue	Source	Ref.
1.	LVVYPWTQRF	32-41		porcine hypothalamus	[4]
				human liquor	[2]
		31-40		bovine hypothalamus	[7]
			3.0	bovine subcortex	
2.	LVVYPWTQR	32-40		human pituitary gland	[5]
3.	LVVYPWTQ	31-38	1.5	bovine subcortex	
4.	LVVYPWT	31-37	0.6	bovine subcortex	
5.	LVVYPW	32-37		porcine bone marrow	[6]
6.	LVVYP	31-35	0.3	bovine subcortex	
7.	VVYPWTQRF	32-40	0.4	bovine subcortex	
		33-41		porcine hypothalamus	[4]
8.	VVYPWTQ	32-38	1.0	bovine subcortex	
				bovine hypothalamus	[1,7]
9.	VVYPWTQR	32-39		bovine hypothalamus	[7]
10.	VYPWT	33-37		bovine hypothalamus	[7]
11.	VYPWTQ	33-38		bovine hypothalamus	[7]

Earlier, the indirect evidences of isolated peptides endogeneity were published [8]. In particular, one of the arguments is a regularity of the isolation of hemorphin-containing fragments of hemoglobin from different biological sources, such as porcine hypothalamus [4] and bone marrow [6],bovine subcortex [8] and human pituitary gland [5]. Besides, there is a natural distribution of hemoglobin peptide fragments in brain tissue. The comparison of the isolated hemorphin-containing peptides from different sources shows that they differ by both their compositions and their tissue-containing levels. For example, among the six

Table 2. Opioid-like fragments derived from enzymatically treated functional proteins

Structure	Name	Source	Ref.
Y P F V E P I P Y	Casomorphin-9	β-casein 75-83	[9]
Y P F V E P I P	Casomorphin-8	β-casein 75-82	[9]
Y P F V E P I	Casomorphin-7	β-casein 75-81	[9]
Y P F V E P	Casomorphin-6	β-casein 75-80	[9]
Y P F V E	Casomorphin-5	β-casein 75-79	[9]
Y P F V	Casomorphin-4	β-casein 75-78	[9]
Y P F T I	Cytochrophin-5	cytochrome B 345-349	[10]
Y P F T	Cytochrophin-4	cytochrome B 345-348	[10]
L V V Y P W T Q R F	LVV-hemorphin-7	β-hemoglobin 31-40	[21]
V V Y P W T Q R F	VV-hemorphin-7	β-hemoglobin 32-40	[21]
Y P W T Q R	Hemorphin-6	β-hemoglobin 34-39	[5]
Y P W T Q	Hemorphin-5	β-hemoglobin 34-38	[3]
Y P W T	Hemorphin-4	β-hemoglobin 34-37	[3]

isolated peptides only the fragment 31-38 is mainly localized in bovine brain whereas the rest of peptides are in subcortex [14]. The fragment 31-39 is found at high concentration in hypophysis [5]. At the same time neither the summary brain tissue nor brain structures (cortex and subcortex) contain this fragment in comparable amounts. It should be noted that there is a sharp rise in the content level of fragment 31-40 in cerebrospinal fluid of patients with cerebrovascular bleedings [2]. The facts show that the formation process of these peptides has to some extent a tissue specific nature.

The high content of isolated peptide fragments might be explained by resistance to a nonspecific proteolysis of the central part (Tyr-Pro-Trp) of peptide, which is inherent in all hemorphin-containing peptides, but the presence of fragment 31-35 allows to suppose the possibility of specific peptide inactivation *in vivo.*

The opioid activity of hemorphins and peptide 31-39 was investigated by the 3 basic tests: an inhibition of opioid ligands binding; an induction of analgesia in rats; and contractile activity (guinea pig ileum) testing [1,2,5]. Peptides exhibited a positive activity in all these test-systems. The binding constant of hemoglobin fragment 31-39 with μ-opioid receptor is 270 nM [5]. It is considerably greater than that for "classical" opioid ligand, such as Met-enkephalin (about 1.0 nM) [15], but significantly high content of hemorphin-containing peptides *in vivo* makes them effective ligands for opioid receptors. For example, the content of enkephalins in brain is about pmols per gram of brain tissue [16]. It is considerably less than that for hemorphin-containing peptides. Thus, total opioid potentiality of hemoglobin fragment may be comparable with those for "classical" opioid peptides.

At the same time, endogenous hemoglobin fragments exhibit not only opioid activity. It is shown that the fragment 31-39 exhibits an angiotensin converting enzyme activity as well [17]. A number of hemorphin-containing fragments isolated from bovine hypothalamus, such as the fragment 31-40, exhibit a coronaro-constrictory activity [7]. It is necessary to note that the hemoglobin molecule is a source of endogeneous peptides not structurally connected with the hemorphin group. These peptides regulate different organism functions and exhibit an activity in many tests. For example, an alteration of neo-kyotorphin level is a regulating factor of hibernation [18]. It also stimulates a calcium action current along the frog heart muscular fibre, raises a calcium intracellular concentration in rat cardiocytes [18,19].

Neuropeptide-like substances generation is not an unique property of hemoglobin. Pepsin treatment of the blood plasma proteins *in vitro* results in neurotensin-like peptide generation with micromolar concentrations [20]. It allows to suppose that the proteolytic degradation of functional proteins is a significant part of the total peptidergic regulation in organism. It is possible that it takes part in the regulation of long term physiological processes.

References

1. Erchegyi J., Kastin A.J., Zadina J.E., and Qiu X.-D. (1992) Int.J.Pept.Prot.Res.,39,477-484.

2. Glamsta E.-L., Meyrson B., Silberring J., Terenius L., and Niberg F. (1992) Biochem.Biophys.Res.Com., 184, 1060-1069.
3. Brantl V., Gramsch C., Lottspeich F., Mertz R., Jaeger K.- H., and Herz A. (1986) Eur. J.Pharmacol., 125, 309-310.
4. Chang R.C.C., Huang W.-Y., Redding T.W., Arimura A., Coy D.H.,and A.V. Schally. (1980) Biochem. et Biophys. Acta, 685, 266-273.
5. Glamsta E.-L., Marklund A., Hellman U., Wernstedt C., Terenius L., and Nyberg F. (1990). Reg.Pept., 34, 169-179.
6. Fonina L.A., Gur`yanov S.A., Nazimov I.V., Yanovsky O.G., Zacharova L.A., Michailova A.A., and Petrov R.V. (1991) Doklady Academii Nauk, 319, 755-757 (in Russian).
7. Galoyan A.A., Gurvits B.Ya., and Barkhudaryan N.A. (1994) Proceedings of the Eur.Neurochem.Soc.Biannual Meeting, Jerusalem (in press).
8. Ivanov V.T., Karelin A.A., Ul`yashin V.V., Karelina E.V., Mikhaleva I.I., Vaskovsky B.V., Nazimov I.V., Grishina G.A., Khavinson V.Kh.,Morozov V.G., and Mikhaltsov A.N. (1991) In "Peptides 1990."(E.Giralt and D.Andreu,eds.), pp. 813-815. ESCOM, Leuden.
9. Brantl V. (1984) Eur. J. Pharmacol., 106, 213-214. K.-H., Herz A. (1985) Eur. J. Pharmacol., 111, 309-310.
10. Brantl V., Gramsch Ch., Lottspeich F., Henschen A., Jaegger K.-H., Herz A. (1985) Eur. J. Pharmacol., 111, 309-310.
11. Davis T.P., Gillespie T.J., and Porreca F. (1986) Peptides, 10, 747-751.
12. Takagi H., Shiomi H., Ueda H., and Amano H. (1979) Nature, 282, 410-412.
13. Takagi H., Shiomi H., Fukui K., Hayashi K., Kiso Y., and Kitagawa K. (1982) Life Sci., 31, 1733-1736.
14. Ivanov V.T., Karlin A.A., Mikhaleva I.I., Vaskovsky B.V., Sviryaev V.I., and Nazimov I.V. (1992) Bioorg.Khim., 18, 1271-1311 (in Russian).
15. Pasternak G.W. (1981) Neurology, 31, 1311-1315.
16. Ross M., Chararossian V., Cox B.M., and Goldfiin A. (1978) Life Sci., 22, 1123-1130.
17. Lantz I., Glamsta E.-L., Talback L., and Niberg F. (1991) FEBS Lett., 287, 39-41.
18. Vaskovsky B.V., Ivanov V.T., Mikhaleva I.I., Kolaeva S.G., Kokoz Yu.M., Svieryaev V.I., Ziganshin R.H., Sukhova G.S., Ignatiev D.A. (1990) In "Peptides. Chemistry, Structure and Biology"(E.Rivier and G.Marshall ,Eds.),pp. 302-304. ESCOM, Leiden.
19. Svieryaev V.I., Vaskovsky B.V., Ziganshin R.H., Mikhaleva I.I., Ivanov V.T., Kokoz Yu.M., Povzun A.A., Alekseev A.E., and Sukhova G.S. (1991) In "Peptides 1990."(E.Giralt and D.Andreu, Eds.), pp.751-752. ESCOM,Leiden.
20. Carraway R.E., Mitra S.P., and Ferris C.F. (1986) Endocrynology, 119, 1519-1526.
21. Piot J.-M., Zhao Q., Guillochon D., Ricart G., and Thomas D. (1992) Biochem.Biophys.Res.Com., 189, 101-110.