Vol. 118, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 13, 1984 Pages 299-303

# Purification and Sequence of an Opioid Peptide Derived from Ovine Proenkephalin

Radmila Micanovic, Purnima Ray, William Kruggel, and Randolph V. Lewis*

Department of Biochemistry, University of Wyoming, Laramie, Wyoming 82071

Received December 1, 1983

An enkephalin-containing peptide originating from ovine adrenal proenkephalin has been purified and sequenced. The sequence of the peptide is: GLY-GLY-GLU-VAL-LEU-GLY-LYS-ARG-TYR-GLY-GLY-PHE-MET (preproenkephalin 128-140) which represents a portion of peptide F (preproenkephalin 107-140). This peptide has a sequence identical to that of bovine preproenkephalin 128-140 while it differs from the corresponding human sequence in positions 129, 131 and 133.

Since the discovery of Met- and Leu-enkephalin by Hughes et al. (1) a number of other endogenous opioid peptides have been characterized in mammalian tissues (2). All of them are derived from one of three separate precursors: pro-opiomelanocortin (3), proenkephalin (4,5) or prodynorphin (6). The adrenal medulla is known to be one of the richest sources of endogenous opioid peptides, containing especially high concentrations of peptides derived from proenkephalin (7-10). The mRNA coding for that prohormone was isolated, cloned and sequenced from bovine adrenal medulla and human pheochromocytoma tissue (4,5). Many of the proenkephalin fragments, both of opioid and non-opioid nature, have been isolated and sequenced (10-12). Nonetheless, many steps involved in the precursor processing in the adrenal gland remain to be elucidated.

We report here the purification and characterization of an internal proenkephalin peptide from ovine adrenal chromaffin granules. This peptide is the 128-140 residues of the bovine preprohormone sequence, representing the carboxy terminal portion of peptide F.

## Materials and Methods

Chromaffin granules were prepared from fresh ovine adrenal medullas by differential centrifugation as described elsewhere (13). The granules were

*To whom correspondence should be addressed

0006-291X/84 $1.50

lysed in 1M acetic acid-20 mM HCl containing 0.1% 2-mercaptoethanol and 1μg/ml each of phenylmethanesulfonylfluoride and pepstatin A as protease inhibitors. The final supernatant from the 40,000 xg centrifugation (1 hr) was chromatographed on a Sephadex G-100 column (5x100 cm) with lysis buffer lacking protease inhibitors as an eluant. Column fractions were pooled by molecular weights according to previous work (7) yielding five peaks.

The high performance liquid chromatography system (14) was used with acetic acid/pyridine buffer (0.5M/0.2M, pH 4) and 1-propanol as the organic modifier. The conditions of each chromatographic procedure are given in the figure legends.

Radioimmunoassays were performed with an antiserum obtained from Immunonuclear (Stillwater, MN) using [$^{125}$I] Met and Leu-enkephalin (New England Nuclear, Boston, MA) as described previously (15). Fractions were digested with trypsin and carboxypeptidase B prior to assay (8).

Amino acid analysis were carried out using the o-phthalaldehyde pre-column labeling method (15). A Spectra Physics 8700 HPLC system and a Schoeffel/Kratos 950 fluorometer were utilized with an Alltech 3μ $C_{18}$ column (4.6 x 150 mm). The gradient was 50 mM Na acetate (pH 6.6) to 80% methanol in the same buffer with 1% tetrahydrofuran in both.

Peptide sequence analysis was performed on approximately 600 pmoles of the peptide using the Applied Biosystems model 470A protein sequencer as described by Hewick, et al. (17). Phenylthiohydantoin amino acids were identified by HPLC (Beckman 344 HPLC system) using a Bakerbond $C_{18}$ column with the trifluoracetic acid-acetic acid/acetonitrile buffer system of Hawkes et al. (18) using absorbance at 269 nm for detection.

## Results

Following chromatography of the chromaffin granule extract on Sephadex G-100 the fractions corresponding to peak IV (MW=1000-5000) were pumped onto an Alltech 10μ $C_{18}$ column (Figure 1A). Fractions 17-18 (48-54 min) were pooled, vacuum evaporated and rechromatographed on a $C_{18}$ HPLC column with a shallow gradient yielding a single sharp peak (Figure 1B). This peak was then chromatographed on a diphenyl column (19), that has markedly different selectivity, using a shallow gradient (Figure 1C). A single symmetric peak was also obtained with this column. The peptide (fractions 17-19) was vacuum evaporated, injected onto a clean $C_8$ column, and concentrated in a small volume (200 μl) by pulsing off the column with 40% 1-propanol. Amino acid analyses and sequencing were performed on that sample.

It should be noted that each HPLC purification step was followed by radioimmunoassay of the peak fractions and in all instances enkephalin immunoreactivity was observed, as can be seen in Figures 1B-C.

Amino acid analysis data are presented in Table I indicating a small peptide with the composition $Gly_5$, Glu, Val, Leu, Lys, Arg, Tyr, Phe, Met. Sequence analysis revealed a sequence of GLY-GLY-GLU-VAL-LEU-GLY-LYS-

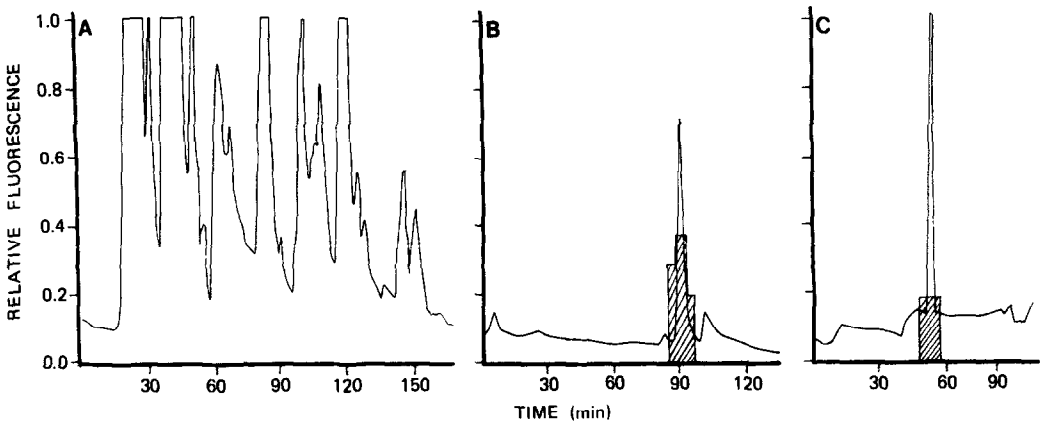


Figure 1. Purification of Ovine Preproenkephalin 128-140. (A) Fractions from the Sephadex G-100 column corresponding to molecular weights of 1000-5000 were pooled and pumped directly onto an Alltech 10 μ $C_{18}$ column. The peptides were eluted with a gradient of 1-propanol in 0.5M acetic acid brought to pH 4.0 with pyridine. The gradient was: 0%-21.33% 1-propanol in 120 min, 21.3%-32.0% in 60 min, 32.0%-40% in 40 min. (B) Fractions 17-18 (48-54 min) were pooled and vacuum concentrated. The sample was resuspended in 1 ml of starting buffer and loaded on a Bakerbond 5 μ $C_{18}$ column. The buffers were as in A with the gradient being: 0%-9.3% 1-propanol in 120 min, 9.3%-40% in 60 min. (C) Fractions 30-32 (87-96 min) were pooled and vacuum concentrated. The sample was resuspended in 1 ml as in B and injected on a Bakerbond Diphenyl column. The buffers were as in A with a gradient of: 0%-10.6% 1-propanol in 120 min, 10.6%-40% 1-propanol in 60 min. Fractions 17-19 (48-57 min) were pooled and used in further analyses. The solid trace is the fluorescence and the crosshatched area is the activity with the Met-enkephalin radioimmunoassay.

ARG-TYR-GLY-GLY-PHE-MET. This sequence corresponds to residues 128-140 of bovine preproenkephalin (2,4,5,10). It can be seen that this enkephalin containing peptide represents the carboxy terminal portion of Peptide F (Figure 2).

## Discussion

The isolation of this proenkephalin peptide from ovine adrenal is the first from that species. Its sequence demonstrates a complete homology to the

Table I: Amino Acid Analysis

| | Mole Fraction | Number |
|---|---|---|
| Glu | .08 | 1 |
| Gly | .37 | 5 |
| Arg | .08 | 1 |
| Tyr | .08 | 1 |
| Met | .08 | 1 |
| Val | .06 | 1 |
| Leu | .08 | 1 |
| Lys | .08 | 1 |

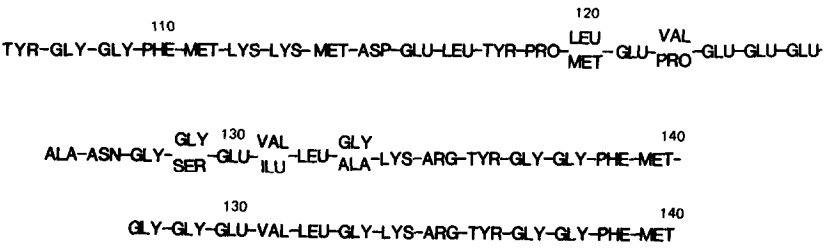


Figure 2. Bovine and Human Peptide F and the Ovine Fragment. The sequence of Peptide F (Preproenkephalin 107-140) is shown with the bovine sequence above and human below where differences are known. The bottom sequence is that of the ovine sequence (Preproenkephalin 128-140).

bovine sequence and shows no difference at the three positions that differ between human and bovine preproenkephalin in this region (residues 129, 131 and 133) (4,5). Although in an evolutionary sense the bovine and ovine proteins are not greatly separated, the fact that the sequences are identical further suggests that there does exist evolutionary pressure to maintain this sequence since even the residues which have changed in the human are conservative changes.

The fact that this peptide represents only a fragment of Peptide F poses a difficulty. Using this same isolation procedure with bovine adrenals, we have isolated only proenkephalin peptides which were cleaved at basic residues (13,20). We have isolated several other ovine adrenal peptides all of which appear to be cleaved at basic residues (unpublished data). Thus, we have no reason to suspect proteolysis occurred during our isolation. The peptide is a substantial peak on the HPLC and cannot be dismissed as small percentage of Peptide F cleavage. In addition, the probable Lys at residue 141 (from beef proenkephalin) has been removed, presumably by the carboxypeptidase B known to be present in the adrenal medulla (21). At this time we do not know the ovine sequence preceeding this peptide. However, the preceeding residues are conserved in human and bovine proenkephalin and do not contain a basic cleavage site. We are currently isolating and sequencing the larger ovine proenkephalin fragments which should enable us to determine the origin of this peptide and its cleavage site.

## Acknowledgements

We would like to thank Jacque Watkins for preparation of this manuscript. This research was supported by NIH grant NS 17647-03 (to RVL) and the DOD University Instrumentation Program (sequencer).

## References

1) Hughes, J., Smith, T.W., Kosterlitz, H.W., Fothergill, L.A., Morgan, B.A., and Morris, H.A., (1975) Nature 258, 577-79.
2) Lewis, R.V. and Stern, A.S. (1983) Ann. Rev. Pharm. Toxic 23, 353-372.
3) Nakanishi, S., Troul, A., Kita, T., Nakamura, M., Chang, A.C.Y., Cohen, S.N., and Numa, S., (1979) Nature 278, 423-427.
4) Noda, M., Furutani, Y., Takahashi, H., Toyosato, M., and Hirose, T. (1982) Nature 295, 202-206.
5) Comb, M., Seeburg, P., Adelman, J., Eiden, L., and Herbert, E., (1982) Nature 295, 663.
6) Kakidani, H.,Furutani, Y., Takhashi, H., Noda, M., Marimoto, Y., Hirose, T., Asai, M., Tnayama, S., Nakanishi, S., and Numa, S. (1982) Nature 298, 245-49.
7) Lewis, R.V., Stern, A.S., Rossier, J., Stein, S., and Udenfriend S. (1979) Biochem. Biophys. Res. Comm. 89, 822.
8) Lewis, R.V., Stern, A.S., Kimura, S., Rossier, J., Stein, S., and Udenfriend, S., (1980) Science 208, 1459-61.
9) Lewis, R.V., Stein, S., Gerber, L.D., Rubenstein, M., and Udenfriend, S. (1978) Proc. Natl. Acad. Sci. USA 75, 4021.
10) Jones, B.N., Stern, A.S., Lewis, R.V., Kimura, S., Stein, S., and Udenfriend, S. (1980) Arch. Biochem. Biophys. 204, 392-395.
11) Jones, B.N., Shively, J.E., Kilpatrick, D.L., Stein, A.S., Lewis, R.V., Kojima, K., and Udenfriend, S. (1982) Proc. Natl. Acad. Sci. USA 79, 2086-2100.
12) Lewis, R.V., Ray, P., Blacher, R., and Stern, A., (1983) Biochem Biophys. Res. Comm. 113, 229-243.
13) Evangelista, R., Ray, P., and Lewis, R.V. (1982) Biochem. Biophys. Res. Comm. 106, 895-902.
14) Lewis, R.V. (1979) Anal. Biochem. 98, 142-145.
15) Lewis, R.V. (1982) Adv. in Biochem. Psychopharm. 33, 167-174.
16) Jones, B.N., Paabo, S., and Stein, S. (1981) J. Liq. Chromat. 4, 565-586.
17) Hewick, R.M., Hunkapiller, M.W., Hood, L.E., and Dreyer, W.J. (1981) J. Biol. Chem. 256, 7990-7997.
18) Hawke, D., Yuan, P-M., and Shiveley, J.F. (1982) Anal. Biochem. 120, 302-311.
19) Stern, A.S., Lewis, R.V., Kimura, S., Rossier, J., Stein, S., and Udenfriend, S. (1980) Arch. Biochem. Biophys. 205, 606-613.
20) Lindberg, I., Yang, H-Y.T., and Costa, E. (1982) Biochem. Biophys. Res. Comm. 106, 186-193.
21) Fricker, L.D. and Snyder, S.H. (1982) Proc. Natl. Acad. Sci. USA 79, 3886-3890.