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MASS SPECTROMETRIC IDENTIFICATION OF VARIOUS MOLECULAR FORMS OF DYNORPHIN IN BOVINE ADRENAL MEDULLA

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SUMMARY: Two dynorphin-(Dyn) immunoreactive fractions (I and II) were isolated from bovine adrenal medulla. On Fast Atom Bombardment (FAB) mass spectrometry, fraction I was identified as Dyn-(1-12) and fraction II was shown to be a mixture of Dyn-(1-11), Dyn-(1-13) and two modified forms of Dyn-(1-13) with molecular weights exceeding that of the parent peptide by 14 and 28, respectively. These results suggest that the Dyn-biosynthetic pathway may generate different types of biologically active molecules.

The adrenal medulla is one of the major sources of endogenous opioid peptides[1-7]. In this tissue, the levels of Leu-enkephalin, Met-enkephalin, enkephalin-containing hexapeptides and heptapeptides are 5 to 15- old higher than those found in the brain striatum[7]. High molecular weight precursors have also been identified and shown to generate several copies of both Leu- and Met-enkephalin under the proper proteolytic cleavage[8]. Finally, the complete identification of the cDNA structure of the gene coding for bovine adrenal preproenkephalin[9] support the concept of common precursors for both Leu- and Met-enkephalin but not for α-neoendorphin [10] nor dynorphin[11]. Recent studies in our laboratory demonstrated the presence of immunoreactive-Dyn in glandular and cellular extracts of bovine adrenal medulla[12] and its secretion from isolated adrenal chromaffin cells[13]. The purification of this material allowed the isolation of two distinct fractions (fraction I and II) which were shown to be as active as synthetic Dyn-(1-13) in the guinea

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pig ileum assay. Herein, we present the mass spectrometric identification of various molecular forms of Dyn in peptide fractions obtained from the bovine adrenal medulla.

MATERIAL AND METHODS

Two hundred bovine adrenal glands were collected at a local slaughterhouse and the Dyn-immunoreactive material was extracted from the medullary tissue as previously described[12]. Briefly, the medullae were dissected free of cortical tissue and submitted to a 0.1 M HCl-0.1% thiodiethanol extraction at 95°C for 15 min. After neutralization at 0°C, the immunoreactive Dyn in the soluble material was purified by chromatography on Sephadex G-10, carboxymethylcellulose, Sephadex G-50 and partition chromatography on Sephadex G-50.

Two immunoreactive fractions were collected from the latter purification step (fractions I and II). Each fraction was then submitted to high pressure liquid chromatography on μ-Bondapak C18 reverse phase column (HPLC, Fig. 1). The Dyn-immunoreactivity was monitored by radioimmunoassay using a highly specific and sensitive antiserum to Dyn-(1-13)[13] (Fig. 1B). The elution pattern of each HPLC chromatogram showed several peaks of optical density monitored at 214 nm (Fig. 1A), with one major peak being immunologically reactive (Fig. 1B). Sample I (tubes 27-28, HPLC fraction I) and sample II (tubes 23-26, HPLC fraction II) were then collected to yield 4 and 6 μg of material, respectively.

For analysis by mass spectrometry, samples were dissolved in 5% acetic acid and 1-2 μl loaded onto the probe tip, previously coated with 5 μg of glycerol[14]. Fast Atom Bombardment spectra were recorded on a VG ZABIF instrument using an M-SCAN FAB gun operating at 8KV and 25 μA. Derivatives were prepared by dissolving 1 μg of peptides in 25 μl of water and adding 20 μl of reagent made up from acetic anhydride: d_6 acetic anhydride 1:1(V/V) in methanol, the anhydride to methanol ratio being 1:3(V/V). Tryptic digestion of samples I and II was carried out using 1 μg of substrate in 0.1 M amonium bicarbonate, pH 8.5 for 1h at 37°C, at an enzyme:substrate ratio of 1:50.

RESULTS

The identification of Dyn in samples I and II was made by mass spectrometry. The FAB analysis of 1 μg of sample I showed a strong quasimolecular ion at m/e 1475 with little fragmentation (Fig. 2A). This molecular weight corresponds to that predicted for Dyn-(1-12): Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu. The validity of this conclusion was checked in two ways. Firstly, that the signal corresponded to a peptide with a free amino-terminus was demonstrated by the observation of a shift to give a doublet 1:1 signal after acetylation at m/e 1517-1520. Secondly, and importantly, following tryptic digestion and direct analysis of the mixture by FAB, the spectrum shown in Fig. 2B was generated. The data can be rationalized with possible expected tryptic fragments from Dyn-(1-12), namely Tyr-Gly-Gly-Phe-Leu-Arg at m/e 712, its companion fragment Arg-Ile-Arg-Pro-Lys-Leu at m/e 782; Tyr-Gly-Gly-Phe-Leu-Arg-Arg at m/e 868 and its companion fragment Ile-Arg-Pro-Lys-Leu at m/e 626. No other major sig-

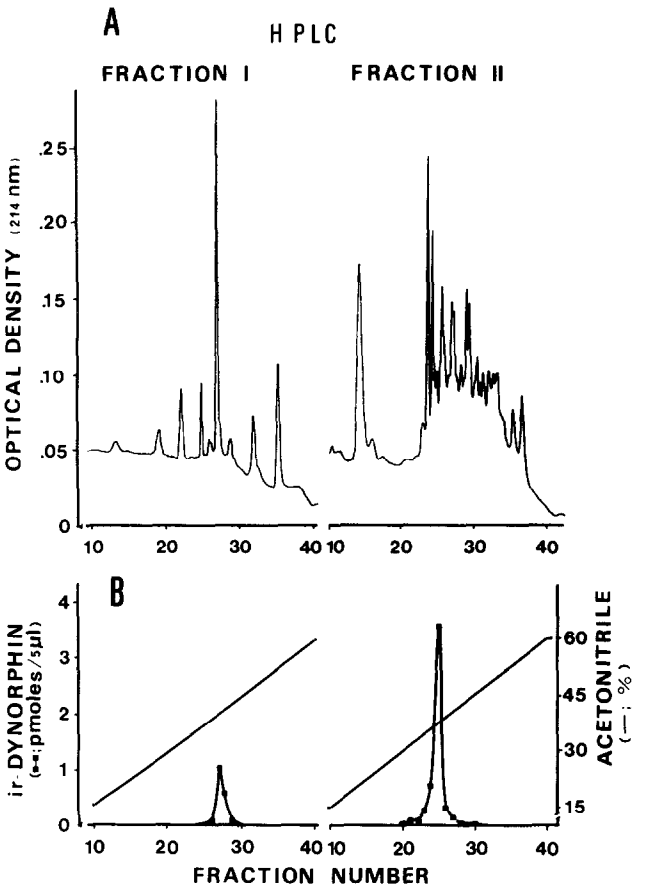

Fig. 1 (A) High pressure liquid chromatography (HPLC) of two dynorphin-immunoreactive fractions obtained from the chromatography by partition. HPLC was performed with a Waters µ-Bondapak C18 reverse phase column (3.9 x 300 cm). The column was eluted with 0.08% trifluoroacetic acid (pH 2.4) with a linear gradient of acetonitrile from 15% to 60% within 40 min at a flow rate of 1 ml/min. 1 ml-fractions were collected. Absorbance was recorded at 214 nm with model 441 absorbance detector (Waters). (B) Immunoreactive Dyn measured by radioimmunoassay on 10 µl samples. The radioimmunoassay was performed as described by Day et al.[13] Sample I (tubes 28, 29) and sample II (tubes 23-26) were collected from the HPLC on fraction I and II, respectively.

nals are left uninterpreted in the spectrum and taken together with the Dyn immunoreactivity these physicochemical data provide convincing evidence that sample I is Dyn-(1-12).

The second fraction possessing Dyn immunoreactivity (sample II, Fig. 1) was examined as the free peptide (2 µg) by FAB. This time, the data demonstrated the presence of a mixture of peptides, having quasimolecular ions at m/e 1362, 1603, the latter signal also carrying satellites at m/e 1617 and 1631 (Fig. 3A). The signal at m/e 1362 would correspond in mass to Dyn-(1-11): Tyr-Gly-Gly-Phe-

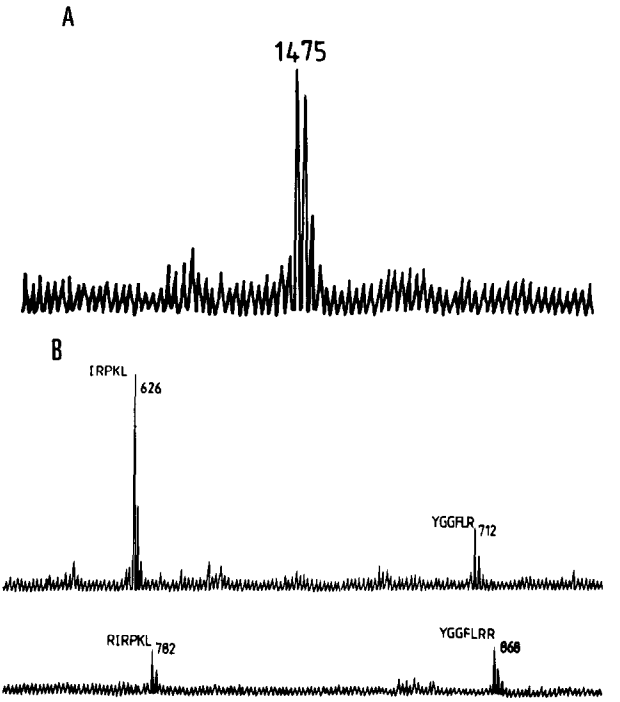

Fig. 2 A. Molecular ion region of the FAB mass spectrum of 1 μg of sample I. B. FAB mass spectrum of a tryptic digest of 1 μg of sample I.

Leu-Arg-Arg-Ile-Arg-Pro-Lys whereas that at m/e 1603 would correspond to Dyn-(1-13) itself: Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys. The remainder of the sample was split for two experiments: the first 2 μg was treated with trypsin and examined as the free peptide mixture. The second 2 μg of tryptic digest was then 1:1 acetylated prior to the FAB experiment. The spectrum of the free digest product is shown in Fig. 3B. In the free spectrum a signal at m/e 712 is assigned to Tyr-Gly-Gly-Phe-Leu-Arg and its companion fragment is present as a signal at m/e 669: Arg-Ile-Arg-Pro-Lys. m/e 868 is assigned to Tyr-Gly-Gly-Phe-Leu-Arg-Arg and its companion signal is also present at m/e 513 for Ile-Arg-Pro-Lys. The dipeptide Leu-Lys is observed at m/e 260.

Interestingly, 14 mass unit satellites were observed at m/e 1617 and 1631 in the intact sample II experiment (Fig. 3A) and these satellites are now accompanying the N-terminal digest fragments assigned to Tyr-Gly-Gly-Phe-Leu-Arg at m/e 712 and Tyr-Gly-Gly-Phe-Leu-Arg-Arg at m/e 868, but are not observed with any of the C-terminal fragments (Fig. 3B). These data suggested the presenc

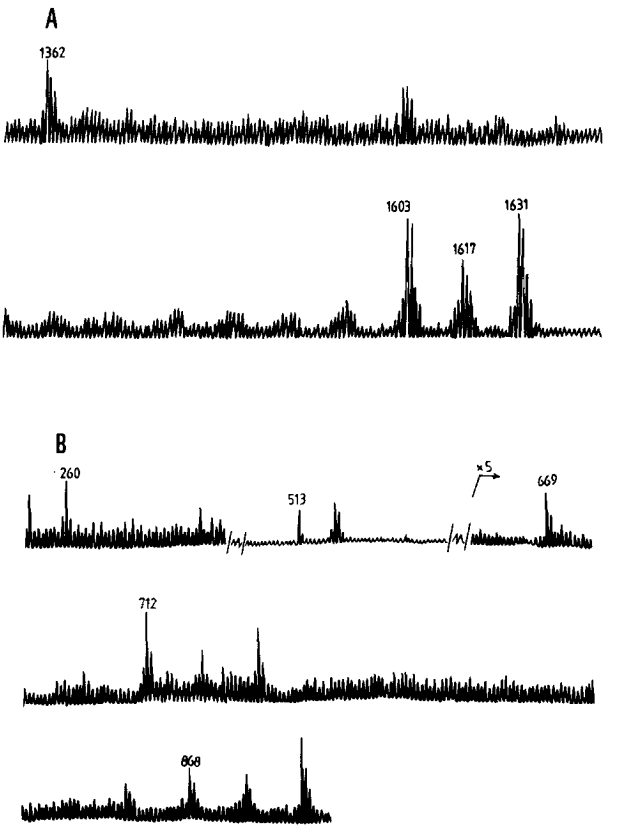

Fig. 3 A. Molecular ion region of the FAB mass spectrum of 2 μg of sample II.
B. FAB mass spectrum of a tryptic digest of 2 μg of sample II.

in sample II of either N-methyl analogues or of homologous amino-acid replacement such as Ala for Gly in the Dyn sequence. Some possibilities can be discounted, for example a methyl group cannot reside on Arg-7 since the same satellites are present at m/e 712, Dyn-(1-16). Similarly, in the 1:1 acetyl spectra the same two satellites are observed for the corresponding signals at m/e 754 and 910; the formation of these derivatives discounts the possibility of N-bis methyl Tyr_1. The most likely interpretation of the date would appear to be the presence in sample II of Dyn sequences containing Enk structure with single and double replacements of Ala for Gly. However, other alternatives could also be considered as the mono methylation of Tyr, or replacement of some amino acid between position 1 and 6 which would give rise to the proper molecular forms.

DISCUSSION

The location and structural identification of Leu- and Met-enkephalin and enkephalin-containing peptides in the adrenal medulla has been well documented[1-8]

However, relatively little is known about the biosynthesis and/or secretion of Dyn and Dyn-containing peptides or fragments at this level. Recent studies in our laboratory have demonstrated the presence of immunoreactive Dyn in glandular and cellular extracts from the bovine adrenal medulla[12,13]. The immunoreactive material was purified and separated on partition chromatography into two active fractions (I and II)[12]. HPLC and mass spectrometry analyses revealed the presence of Dyn-(1-12) in fraction I (Fig. 2) and that of a mixture of Dyn-(1-11), Dyn-(1-13) and two modified forms of Dyn-(1-13) in fraction II (Fig. 3). The various molecular forms of Dyn could have been generated during the extraction procedure. However, our first purification step, consisting in pouring the medullary tissue in a 95°C-HCl solution for 15 min, should eliminate or at least greatly minimize the endogenous proteolytic activity. Hydrolysis of standards under these conditions is negligible.

Previous studies with the porcine pituitary and the porcine duodenum have identified Dyn as an opioid peptide of 17 residues[11,16,17]. The N-terminal sequence of 13 of its amino acids was first obtained[11], but recently, the whole structure of the peptide was also elucidated[16,17]. Our extraction products did not apparently contain Dyn-(1-17). However, since our antiserum was highly specific for Dyn-(1-13)[13], we may have lost the heptadecapeptide during the extraction procedure. Conversely, the fact that Dyn-(1-13), Dyn-(1-12) and Dyn-(1-11) have reportedly been shown to be almost as active as Dyn-(1-17)[1] may indicate that the shorter peptides exist as such in the adrenal medulla and are released upon cholinergic nerve stimulation[12].

Another interesting aspect of our study is the identification of naturally modified forms of Dyn-(1-13). The mass data obtained with the acetylated and tryptic peptides indicate that the modifications are located between residues 1 and 6 in the sequence and are assigned on the basis of mass, either to natural N-methylation or, more likely, the existence of mono- and/or di-Ala-containing Dyn. Kimura *et al.*[8] first demonstrated the presence in the adrenal medulla of enkephalin-containing peptides which generate more than one copy of both Leu- and Met-enkephalins when submitted to the proper proteolytic cleavage.

Their data were supported by the elucidation of the complete structure of the gene coding for the enkephalin precursor[9]. Our finding of various molecular forms of Dyn in the bovine adrenal medulla may indicate that the Dyn biosynthetic pathway, similarly to the enkephalin system, generates several forms of biologically active molecules. The particular role of adrenal Dyn as well as that of adrenal Leu- and Met-enkephalins remains to be elucidated. In this regard, Dyn was shown to be the most potent opioid peptide in antagonizing the nicotine-evoked catecholamine secretion from isolated adrenal chromaffin cells, but this effect could not be related to the stimulation of an opiate receptor[19] The characterization and biological role of the opiate binding site at this level[18] is under present investigation.

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