OPIOID PEPTIDE PRECURSORS IN STRIATUM

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1. Introduction

Studies utilising pulse-chase incorporation of radiolabelled amino acids into leucine-enkephalin (LE) and methionine-enkephalin (ME) have indicated that these opioid peptides are derived from the cleavage of ribosomally synthesised precursor protein [1,2]. A number of putative precursor proteins (pro-enkephalins) have been detected in brain [3,4], gut [5] and adrenal medulla cells [6,7]. These proteins are dissimilar to those which act as precursors for the endorphin opioid peptide system, i.e., pro-opiocortin–lipotropin–β-endorphin. We have shown that soluble acid extracts of brain tissue yield proteins of $M_r > 5000$ which have no overt opiate receptor agonist activity but which generate active opioid peptides on digestion with trypsin. We have further shown that sequential enzymatic treatment with trypsin and then carboxypeptidase β yields peptides with chromatographic and immunoreactive properties indistinguishable from ME and LE [8]. Udenfriend's group [9] have obtained similar results for putative pro-enkephalins of the adrenal medulla. This paper describes the further identification of possible precursors for the enkephalins in the guinea-pig striatum.

2. Experimental

2.1. *Extraction of protein*

Guinea pigs (♂, 250–400 g) were killed by cervical dislocation and exsanguination. The brains were removed and the striata dissected out and frozen immediately in liquid nitrogen. The tissue was either used immediately or stored at −70°C, the latter procedure having no apparent effect on the properties of the protein. The frozen tissue was homogenised with 10 ml/g of ice-cold 1 M acetic acid adjusted to pH 1.9 with HCl. The soluble fraction was directly applied to a Sephadex G-100 column after centrifugation for 20–30 min at 18 000 × *g*.

2.2. *Enzyme digestion*

Samples were incubated with 10 μg/ml trypsin (Sigma, type X1, DPCC-treated) for 2 h in 50 mM Tris–HCl, (pH 8.4) containing 1 mM $CaCl_2$. Carboxypeptidase-β (Sigma, C7011, 175 units/mg) digestion was done under the same conditions at 10 μg/ml for 1 h.

2.3. *Chromatography*

The opioid peptides generated by enzymic digestion were routinely isolated by passing the incubation mixture through 100 mg of Poropak Q (Waters Inc.), and after at 10 ml water wash the products were eluted with 5 ml 95% ethanol.

High-pressure liquid chromatography (HPLC) separation and isolation of the enkephalins and other products was carried out on a Whatman reverse-phase column (Partisil ODS-10/25, 250 × 4.5 mm) with an isocratic elution system of ethanol/water/acetic acid (75/25/1, by vol.). Preparative gel filtration was performed on a Sephadex G-100 (4.8 × 83 cm) column eluted with 1 M acetic acid at 4°C.

2.4. *Electrophoresis*

Sodium dodecylsulphate(SDS)–15% polyacrylamide vertical slab-gel electrophoresis was done for 20 h at a constant voltage of 40 V [10,11]. The samples consisted either of fractions from Sephadex G-100 chromatography or the total soluble protein emerging in the void volume from a 20 × 1 cm Sephadex G-25 column. Protein was recovered from the 2 mm sliced gel by incubating the slices for 16 h with 1 ml each of 50 mM Tris–HCl buffer (pH 8.3) at 4°C.

Preparative isoelectric focussing was carried out on a LKB slab-gel system using Ultradex® granular gel

and 2% LKB ampholyte at pH 3–10 and pH 7–10. The gel was run at a constant power of 8 W for 16 h at 12°C. Protein was recovered from the sliced gel by eluting with 3 ml distilled water.

2.5. *Opioid peptide assays*

Bioassay on the mouse vas deferens was done in a 1 ml bath as in [12]. The minimum detectable amount of ME being ~0.5 pmol; samples were classified as having opioid activity only if the inhibitory response was reversed by 900 nM naloxone (Endo Labs).

Radioimmunoassay was done with ME- and LE-directed rabbit antibodies with the appropriate ^{3}H-labelled ligand (Amersham Radiochemicals). Unbound label was removed by adding charcoal and centrifugation. Antibody LE1 at 1/1000 dilution had a 5% cross-reactivity with ME and no cross-reactivity with β-endorphin or α-endorphin up to 10 nM; 50% displacement of [^{3}H]LE by LE was obtained at 1 pmol. Antibody ME1 showed 50% displacement with 8 pmol ME with 1% cross-reactivity to LE.

3. Results

3.1. *Relative molecular mass (M_r) distribution of proenkephalins*

Sephadex G-100 chromatography of acetic acid extracts indicated the presence of several protein peaks which yielded both bioactive and immunoreactive enkephalin-like peptides after enzyme digestion (fig.1). The major species emerge from the column in the size ranges of 50–80 kM_r and 6–12 kM_r. Smaller peaks of activity were detected at 35–40 kM_r and 15–20 kM_r. When the 50–80 kM_r peaks were subjected to isoelectrophoresis 3 separate bands of proenkephalin were identified with pI values of 7.4, 8.4 and 9.1.

SDS gel electrophoresis of total acid-soluble proteins showed the presence of at least 5 proteins which yielded enkephalin-like activity after enzymic digestion (fig.2). The major peak of activity in this case was a 90 kM_r protein. Gel electrophoresis of the 50–80 kM_r peaks from the Sephadex G-100 column confirmed the presence of 90 and 60 kM_r species in this fraction. Identical results were obtained regarding the molecular species separated by gel electrophoresis when either ME or LE immunoassay or total enkephalin bioassay was used to detect the protein bands after enzymic digestion. The 90, 60, 40 and 20 kM_r

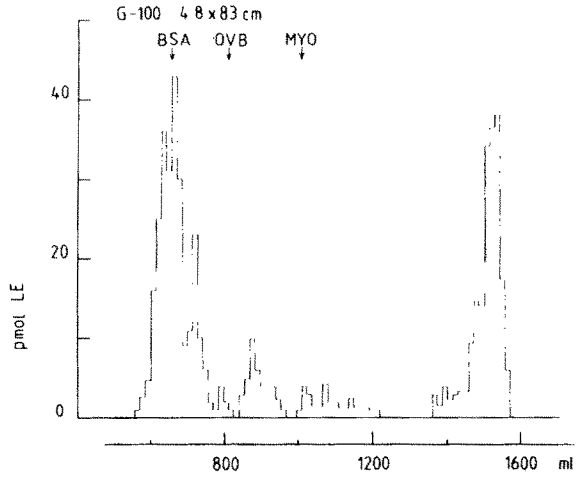

Fig.1. Sephadex® G-100 chromatography of 500 mg acetic acid-soluble striatal protein. Fractions of 15 ml were collected, lyophilysed and sequentially digested with trypsin and carboxypeptidase-B. The histogram shows the amount of LE immunoreactivity generated by each fraction. Control incubations with boiled enzymes showed no activity until after 1650 ml when small endogenous opioid peptides emerge from the column (not shown). The arrows indicate the elution positions of bovine serum albumin (BSA), ovalbumin (OVB) and of myoglobin (MYO).

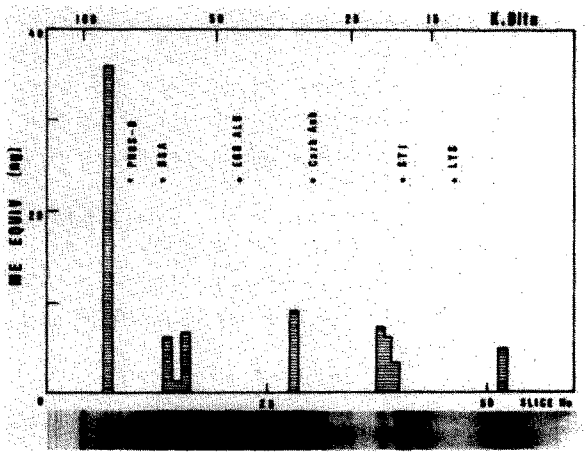

Fig.2. SDS–polyacrylamide gel electrophoresis of 1 mg total soluble protein from void volume of a Sephadex G-25 column. Shaded columns show total bioassayable opioid peptide activity (ng ME equiv.) generated by enzyme digestion of protein in 2 mm slices, no activity was generated by boiled controls. The 5 bands were 92, 60, 40, 20 and ⩽10 app. kM_r as determined by interpolation from the plot of the marker proteins phosphorylase *b*, bovine serum albumin, egg albumin, carbonic anhydrase, soyabean trypsin inhibitor and lysozyme. One track containing 40 μg protein was stained with Coomassie blue (lower panel).

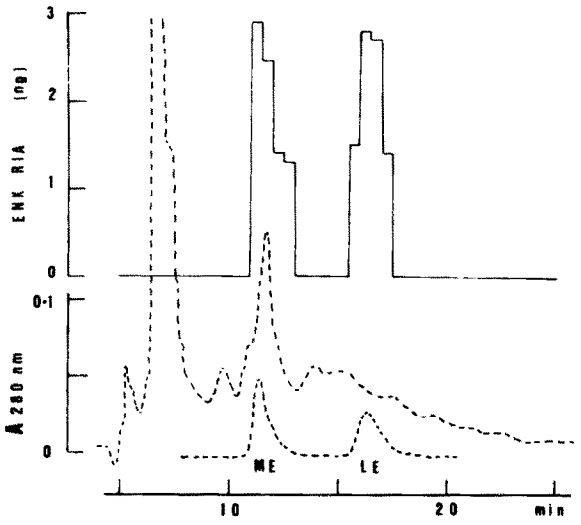

Fig.3. HPLC analysis of enzymic digest of 90 kM_r protein band from gel electrophoresis. The upper solid line shows immunoreactivity detected by LE immunoassay (first peak) and ME immunoassay (second peak). The upper broken line is the A_{280} trace of the sample and the lower trace is the absorbance obtained on injection of 1 μg ME and LE standards.

bands could also be identified by direct immunoassay of the eluted protein with LE1 antibody (1/500 dilution) without any prior enzymic digestion.

3.2. *Chromatographic identification of enkephalins*

The enzymic digests of the protein bands from gel electrophoresis were separated by HPLC and assayed for both LE and ME immunoreactivity (fig.3). These studies confirmed the presence of both ME and LE in proenkephalin. At this time we would be unwilling to assign an absolute value for the ratio of LE/ME in each precursor since there may well be unknown factors influencing the products of enzymic digestion and the recovery of the individual peptides.

4. Discussion

Our identification of the putative pro-enkephalins rests on both immunoassay and bioassay criteria. The results indicate the presence of at least 5 proteins in the guinea-pig striatum which may act as precursors although at present we cannot rule out the existence of a sub-unit structure. It is clear that these pro-enkephalins differ from pro-opiocortin in terms of M_r, immunoreactivity and identity of enzymic products. The apparent complexity of the system, the large M_r of the major species, and the presence of both enkephalins are of considerable interest. The basic nature of the 90, 60 and 40 kM_r proteins is consistent with the presence of paired basic residues providing optimal sites of trypsin cleavage. However proof of a precursor–product relationship must await detailed biosynthetic studies. Also the relationship between the striatal proteins and the smaller adrenal pro-enkephalins remains to be determined.

The large size of the 90 kM_r precursor poses a challenge to analysis although we would predict that this protein may well act as a 'store house' for not only enkephalins and other opioid peptides but also possibly for other biologically active peptides.

Acknowledgements

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