Characterization of new enkephalin-containing peptides in the adrenal medulla by immunoblotting

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Immunoblotting combined with radioimmunoassays (RIAs) directed specifically towards certain sequences of the proenkephalin molecule has been used to characterize the enkephalin-containing peptides (ECPs) present in the bovine adrenal medulla. Immunoblotting allowed the simultaneous visualization of all ECPs present in a crude extract of this gland. Combining this technique with RIAs we have been able to characterize a new high molecular mass ECP, a 23.3-kDa protein which contains the amino-terminal part of proenkephalin and ends with the sequence of Leu-enkephalin at its carboxy-terminus.

Enkephalin *Synenkephalin* *Prohormone* *Adrenal medulla* *Immunoblotting* *Processing*

1. INTRODUCTION

Several enkephalin-containing peptides (ECPs) have been isolated from the bovine adrenal medulla [1–6]. These peptides have been proposed to represent intermediates in the processing of proenkephalin, the polyenkephalin precursor [7–9]. Proenkephalin contains 4 copies of Met-enkephalin and one copy each of Leu-enkephalin, the heptapeptide (Met-enkephalin-Arg^6-Phe^7) and the octapeptide (Met-enkephalin-Arg^6-Gly^7-Leu^8) (fig.1) [10–12]. The sequences of Met-enkephalin, Leu-enkephalin and the octapeptide are bracketed by pairs of basic amino acids (Lys or Arg) and the sequence of the heptapeptide, which is located at the carboxyl terminal of proenkephalin, is preceded by a pair of basic amino acids.

The 3 largest ECPs isolated so far from the adrenal medulla (8.6, 12.6 and 18.2 kDa) have been completely sequenced by classical methods. They all contain the amino-terminal fragment of proenkephalin. The largest molecule (18.2 kDa) contains 3 copies of the Met-enkephalin sequence and ends at the carboxyl terminal with the sequence of the octapeptide. The 12.6- and 8.6-kDa proteins contain 3 and 1 copy of the Met-enkephalin sequence, respectively (fig.1).

Here we have used immunoblotting in combination with RIAs to identify these previously characterized proteins in crude tissue extracts. In addition, two as yet undescribed ECPs were also observed. The largest of these, the 23.3-kDa protein, begins with the amino-terminal sequence of proenkephalin and ends with the sequence of Leu-enkephalin (fig.1).

2. MATERIALS AND METHODS

2.1. *Tissue preparation and electrophoresis*

Bovine adrenal glands were obtained fresh from a local slaughterhouse and transported on ice to the laboratory. The medullae were dissected out and boiled for 20 min in 5 vols of 1 M acetic acid adjusted to pH 1.9 with HCl. The tissue was homogenized with a polytron and centrifuged for 1 h at 20000 × *g*. The supernatant was lyophilized and resuspended in electrophoresis sample buffer. Polyacrylamide gel electrophoresis was performed as in [13] with a stacking gel of 4% acrylamide and

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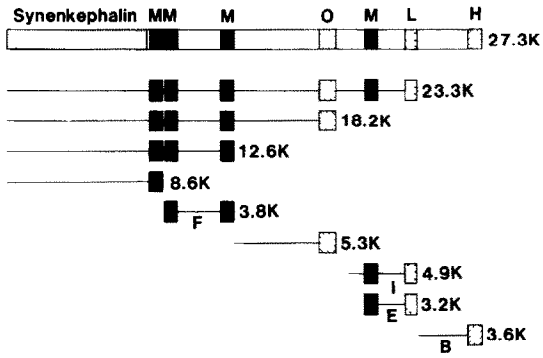

Fig.1. Proenkephalin and the enkephalin-containing peptides in the bovine adrenal medulla. The complete sequence of proenkephalin (27.3 kDa), which is represented schematically on the top of the figure, has been deduced from the corresponding cDNA [10–12]. This protein has never been isolated from the adrenal medulla. The largest ECPs isolated so far, 18.2, 12.6 and 8.6 kDa, contain the N-terminal part of proenkephalin [4–6]. The new 23.3-kDa intermediate characterized here also contains this N-terminal part. Peptides F, 5.3 K, I, E and B are small ECPs which have been purified to homogeneity and sequenced [8,9]. M, Met-enkephalin; O, octapeptide; L, Leu-enkephalin; H, heptapeptide.

a resolving gel of 12% acrylamide, with the addition of 5 M urea in both gels.

2.2. *Immunochemical visualization*

Following electrophoresis, proteins were transferred electrophoretically to nitrocellulose paper in a transblot cell (Bio-Rad) (3 h; 55 V; 0.3 A with cooling to 4°C). The paper was prepared for immunochemical visualization of the antigen essentially as in [14], which included a 10 min rinse in 50 mM Tris–HCl, 200 mM NaCl, 0.02% NaN_3 (pH 7.4) (TBS), followed by a 1 h incubation in the same buffer plus 3% bovine serum albumin (TBSA) to saturate the protein binding capacity of the paper. The paper was cut into strips and incubated overnight at 4°C with 1 of the 3 primary antisera described below. After a 30 min rinse in TBSA, the paper was incubated for 2 h with sheep affinity-purified anti-rabbit IgG which had been coupled to horseradish peroxidase (Institut Pasteur, Paris). Following a 10 min rinse in TBS with the NaCl concentration increased to 0.5 M and a 20 min rinse in TBS, the IgG–peroxidase complexes were visualized by revealing the peroxidase activity with 4-chloro-1-naphthol (3 mM in TBS) in the presence of 0.01% H_2O_2.

The Met-enkephalin and octapeptide antisera used here were directed specifically towards the C-terminal part of the corresponding peptides. The Met-enkephalin antiserum (AC2158) was used at a 1/1000 final dilution and exhibited 10% cross-reactivity with Leu-enkephalin and less than 5% cross-reactivity with the heptapeptide and the octapeptide in RIA [15]. The octapeptide antiserum (AC2227) was used at a 1/1000 final dilution and cross-reacted less than 0.1% with Met-enkephalin, Leu-enkephalin and the heptapeptide in RIA [16]. The synenkephalin antiserum was directed against the N-terminal 1–70 sequence of proenkephalin (synenkephalin) and recognizes neither the Met-enkephalin sequence nor ECPs which do not contain the N-terminal fragment [17]. It was used at a 1/500 final dilution.

2.3. *Proteolytic digestion and extraction of ECPs*

To characterize further the ECPs visualized by immunochemical techniques, lanes of nitrocellulose paper were cut into 2-mm slices. These slices were then treated with trypsin alone (Trypsin TPCK, Worthington; final conc. 100 μg/ml in 0.4 ml of 50 mM Tris–HCl buffer, pH 8.4, containing 2 mM $CaCl_2$; 16 h incubation at 37°C) or trypsin followed by carboxypeptidase B (Boehringer Mannheim; final conc. 0.1 μg/ml in 0.5 ml of 50 mM Tris–HCl buffer, pH 8.4, containing 2 mM $CaCl_2$; 1 h incubation at 37°C). After the trypsin digestion only those enkephalin sequences located at the C-terminal end of ECPs would be expected to be released in a form recognized by our enkephalin antisera, i.e., without C-terminal extension. The other enkephalin sequences released by this treatment would contain C-terminal Arg or Lys residues, and thus would not be recognized by the enkephalin antisera. The subsequent digestion with carboxypeptidase B, which removes the C-terminal basic amino acids, would generate enkephalin. Another lane was cut into 1-mm slices which were treated with protease V8 from *Staphylococcus aureus* (strain V8) which specifically cleaves the peptide bonds at the carboxyl side of glutamic or aspartic acid (Miles Laboratories; final conc. 80 μg/ml in 0.5 ml of 50 mM Tris–HCl buffer, pH 7.8; 16 h incubation at 37°C) [18]. The

digestion with *S. aureus* protease was expected to produce the dodecapeptide Val-Ser-Lys-Arg-Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu from larger ECPs containing at their C-terminal end the sequence of the octapeptide.

2.4. *Radioimmunoassays*

After the proteolytic treatment Leu-enkephalin immunoreactivity (IR) was determined using an antiserum directed towards the C-terminal end of Leu-enkephalin, which cross-reacted less than 5% with Met-enkephalin [19]. Met-enkephalin IR and octapeptide IR were determined using the antisera described above [15,16].

3. RESULTS

3.1. *Characterization of the 23.3-kDa intermediate*

After immunoblotting of a crude adrenal extract, 6 bands corresponding to proteins of 26, 23, 19, 13, 6 and 4 kDa were stained with the Met-enkephalin antiserum (fig.2, lane 1). These bands are numbered 1–6 in fig.2. The protein corresponding to band 1 was also stained by the synenkephalin antiserum, which recognizes all the intermediates containing the N-terminal fragment of proenkephalin (synenkephalin) (fig.2, lane 2). The octapeptide antiserum did not stain this protein (fig.2, lane 3).

To characterize further this protein observed with immunostaining, we used RIA techniques. While digestion of band 1 by trypsin alone did not generate significant amounts of Met-enkephalin-IR, subsequent treatment by carboxypeptidase B enhanced it markedly (fig.3A). Trypsin treatment alone induced the appearance of a large amount of Leu-enkephalin-IR which was not increased further by the subsequent carboxypeptidase B treatment (fig.3B). No octapeptide-IR was detected after treatment of band 1 with the protease of *S. aureus* (fig.4). Therefore, the protein corresponding to band 1, which is stained by both Met-enkephalin and synenkephalin antisera, appears to contain the Leu-enkephalin sequence at its carboxy terminus. From these data and given its apparent M_r, this protein is the largest intermediate in the processing of proenkephalin which has been characterized so far in the adrenal medulla. This protein, proenkephalin (1–206), has a calculated

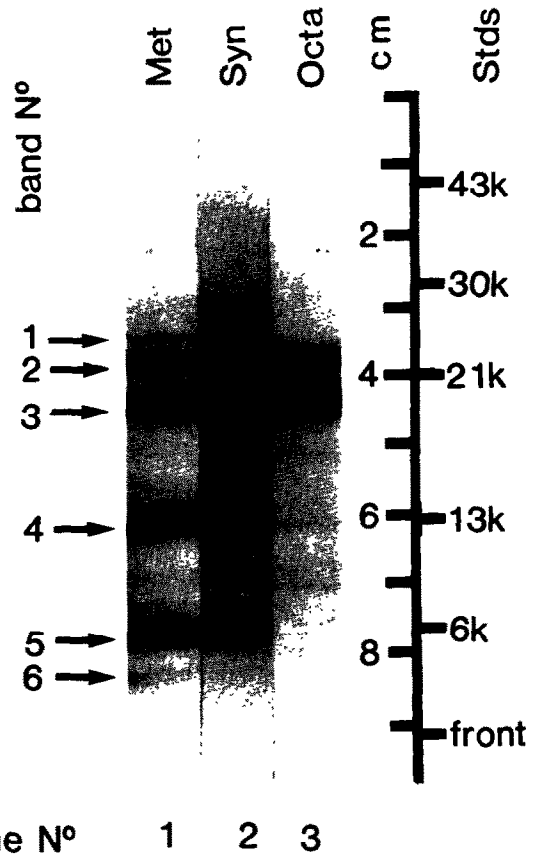

Fig.2. Immunoblotting of a crude adrenal extract. Following electrophoresis the proteins of an adrenal extract were transferred to a nitrocellulose paper. This paper was cut longitudinally in 3 lanes which were stained with anti-Met-enkephalin serum (lane 1), anti-synenkephalin serum (lane 2) and anti-octapeptide serum (lane 3). The standards run in parallel were: ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (21 kDa), cytochrome *c* (13 kDa) and aprotinin (6 kDa).

M_r of 23300.

3.2. *Identification of the 18.2-kDa enkephalin-containing protein*

The proteins corresponding to bands 2 and 3 were stained simultaneously by the Met-enkephalin, octapeptide and synenkephalin antisera (fig.2, lanes 1–3). After processing of the nitrocellulose paper for RIA both bands exhibited substantial amounts of Met-enkephalin-IR but no Leu-enkephalin-IR was detectable (fig.3A and B).

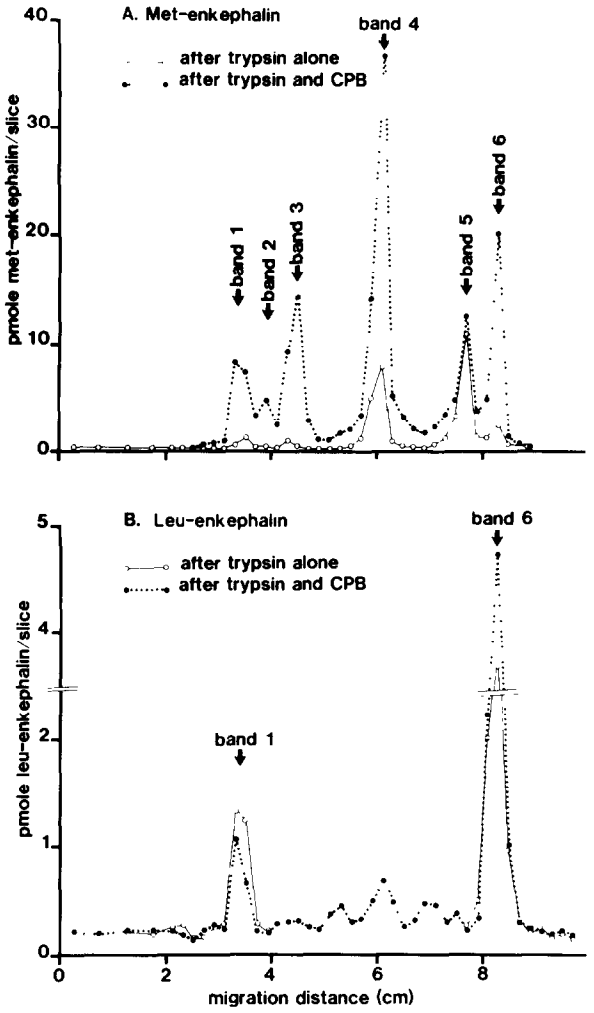

Fig.3. Profile of Met-enkephalin and Leu-enkephalin immunoreactivities following digestion of the immunoblot presented in fig.2. Met- and Leu-enkephalin-IR of 2-mm slices were determined by RIAs after digestion with trypsin alone (○—○) or trypsin followed by carboxypeptidase B (●···●). From the total amount of Met-enkephalin-IR loaded on each lane (0.5 nmol) and the amount recovered after the digestion of the paper with trypsin and carboxypeptidase B (0.18 nmol), an overall yield of 37% has been calculated for the recovery of total Met-enkephalin.

The pattern of octapeptide-IR obtained by RIA after digestion with the protease of *S. aureus* was in good agreement with that obtained by staining. The highest amount of octapeptide-IR corresponded to the protein which exhibited the most intense staining while the other high M_r protein contained

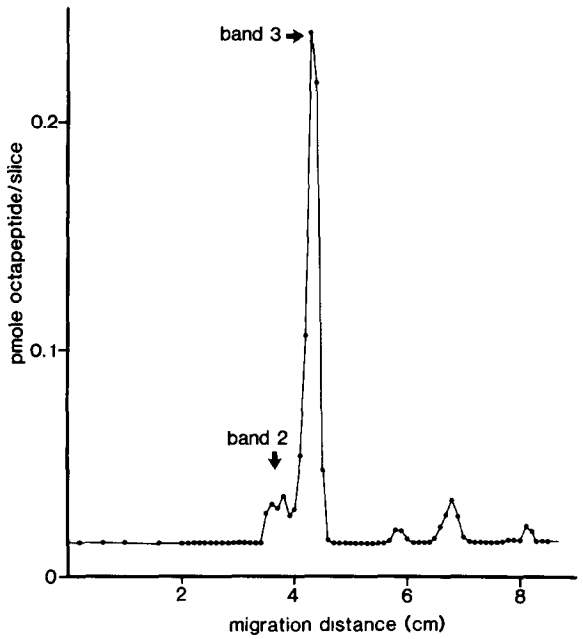

Fig.4. Profile of octapeptide immunoreactivity following digestion of the immunoblot presented in fig.2. Octapeptide immunoreactivity of 1-mm slices was determined by RIA after digestion with *S. aureus* V8 protease.

a much lower amount of octapeptide-IR (fig.4).

Given the high specificity of the octapeptide antiserum towards the C-terminal part of this peptide, it appears from our data that the adrenal medulla probably contains two distinct forms of the previously purified 18.2 kDa intermediate, which contains the N-terminal part of proenkephalin and terminates with the sequence of the octapeptide at its carboxyl end. The intermediate of lower apparent molecular mass (band 3), which is the predominant form, probably represents the 18.2-kDa intermediate reported previously which has been shown not to be glycosylated [20]. The higher molecular mass intermediate (band 2) could conceivably represent a glycosylated derivative of the 18.2-kDa protein, although this clearly needs further support.

3.3. *Visualization of the 12.6- and 8.6-kDa intermediates*

The proteins corresponding to bands 4 and 5 were strongly stained both by Met-enkephalin and synenkephalin antisera. Digestion of these bands

with trypsin alone revealed the presence of a Met-enkephalin sequence at the carboxyl end of both proteins. While the Met-enkephalin-IR of band 4 was increased 280% by further treatment with carboxypeptidase B, indicating that the corresponding protein probably contains 3 copies of Met-enkephalin, the immunoreactivity of band 5 was not changed by this treatment, indicating that the corresponding protein contains a single copy of Met-enkephalin. Given their molecular masses, staining and immunoreactivity patterns, the proteins corresponding to band 4 and band 5 probably represent, respectively, the 12.6-kDa and 8.6-kDa intermediates which have been purified from adrenal medulla [5].

3.4. *Visualization of low* M_r *ECPs*

Band 6 represents in fact two very faint bands corresponding to low-M_r (<6000) peptides. These peptides were stained with the Met-enkephalin antiserum, but not with the synenkephalin antiserum or octapeptide antiserum. These two bands were not resolved by the slicing of the paper and appear after digestion as a large peak of enkephalin immunoreactivity. In this peak Met-enkephalin-IR generated by the digestion with trypsin was increased by further treatment with carboxypeptidase B, while Leu-enkephalin-IR was not changed significantly. Thus, given the pattern of staining, immunoreactivity and the low M_r, these bands probably represent a mixture of small ECPs that may include peptides E, F, I and B.

4. DISCUSSION

Using immunoblotting combined with specific RIAs, we have been able to characterize new high M_r intermediates in the processing of proenkephalin in the bovine adrenal medulla and to visualize simultaneously several other described ECPs. All of the largest ECPs (M_r > 8000) isolated from the bovine adrenal medulla to date have been shown by classical biochemical techniques to contain the N-terminal part of proenkephalin [4–6]. The results obtained by immunoblotting are consistent with these data. All of the largest peptides containing Met-enkephalin immunoreactivity that were detected either by immunoblotting or by RIA are also stained by the synenkephalin antiserum, which is directed specifically towards the N-terminal part of proenkephalin [17].

Furthermore, we have described two new high molecular mass ECPs. The first one contains the sequence of the octapeptide at its C-terminus but appears larger than 18.2 kDa. The other novel peptide extends further towards the C-terminal end of proenkephalin, terminating with the sequence of Leu-enkephalin. These data are consistent with the proposal that the complete processing of proenkephalin in the adrenal medulla involves several successive proteolytic events starting at the carboxyl terminal of this precursor [7–9]. The combination of immunoblotting with specific RIAs appears to be a powerful tool for the study of proenkephalin processing. It allows the simultaneous visualization and characterization of all intermediates present in a crude extract of bovine adrenal medulla, and may be applied to study the products of proteolytic cleavage of proenkephalin by putative processing enzymes in vitro.

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