Identification of a 27-kDa enkephalin-containing protein associated with bovine adrenal medullary chromaffin granule membranes by immunoblotting

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An antiserum which recognizes high molecular mass enkephalin-containing proteins was used to compare proenkephalin intermediates in both the soluble and membrane components of bovine adrenal chromaffin granules by immunoblotting. While a range of molecular mass forms were identified in the soluble lysate the major form in the membranes corresponded to a 27-kDa enkephalin-containing protein. Enzymic digestion of bands of 27-kDa material and quantitation of the enkephalin released showed that 22% of this material was membrane-associated. High concentrations of chaotropic agents were required to extract this material from the membranes. Association of hormone and neuropeptide precursors with membrane components may by important for targeting of precursors to secretory granules or correct processing.

Proenkephalin *Chromaffin granule membrane* *Prohormone packaging* *Immunoblotting*

1. INTRODUCTION

It is clear that most biologically active peptides are synthesized as part of larger precursor molecules [1]. Complex and poorly understood cellular and biochemical events are involved in the conversion of precursors to active peptides. These include the transfer of the prohormone from the rough endoplasmic reticulum to the Golgi apparatus, packaging into secretory granules [2] and limited proteolysis by highly specific proteases [3].

The enkephalin precursor (proenkephalin) which is synthesized in brain and adrenal medullary tissue contains 4 copies of Met-enkephalin and one copy of Leu-enkephalin, Met-enkephalin-Arg^6-Phe^7 and Met-enkephalin-Arg^6-Gly^7-Leu^8 [4,5]. Bovine adrenal medullary tissue contains a number of enkephalin-containing (EC) proteins which represent intermediates in the enkephalin biosynthetic pathway [6]. The largest EC peptides which have been isolated and sequenced correspond to 8.6-, 12.6- and 18.2-kDa N-terminal fragments of proenkephalin. An N-terminal 23.3-kDa fragment has recently been identified by immunoblotting [7].

Several prohormones and some processing enzymes have been shown to be associated with secretory granule membranes [9–11]. We decided to investigate the possibility that proenkephalin may be associated with chromaffin granule membranes. The technique of electroimmunoblotting has been used to characterize proenkephalin present in both lysate and membranes prepared from bovine adrenal chromaffin granules. The antiserum used was generated against a synthetic peptide corresponding to residues 95–117 of proenkephalin and has been shown to recognize high molecular mass EC proteins in bovine adrenal medulla [12].

2. MATERIALS AND METHODS

2.1. *Antisera to proenkephalin(95–117)*

Antisera were raised in rabbits to a synthetic

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peptide corresponding to residues 95–117 of bovine proenkephalin as described [12]. The rabbit 4 antiserum used here had very similar specificity to the rabbit antisera previously characterized. The radioimmunoassay for proenkephalin(95–117) used this antiserum at a final dilution of 1:27000 under the conditions in [12].

2.2. *Preparation of bovine adrenal medullary chromaffin granules*

Bovine adrenal tissue was collected from local slaughterhouses and placed on ice. The medullary tissue was carefully dissected and chromaffin granules prepared as in [13] with the final step employing centrifugation through 1.8 M sucrose to minimize contamination with lysosomes [14]. All procedures were carried out at 4°C. Chromaffin granules were lysed by the addition of 5 ml of 5 mM Tris-succinate, pH 5.9, to each chromaffin granule pellet followed by freeze-thawing. Chromaffin granule membranes were collected by centrifugation at 100000 × *g* for 30 min. The lysate was removed and stored at −70°C. Membrane pellets were resuspended in 5 ml Tris-succinate, pH 5.9, and subjected to 4 additional cycles of freeze-thawing and finally collected by centrifugation at 100000 × *g* for 30 min. Chromaffin granule membrane pellets were washed 3 times with Tris-succinate, pH 5.9, by centrifugation and extracted with 50 mM phosphate buffer, pH 7.5/0.15 M NaCl, 1 M KCl, 1.5 M NaSCN, 1 M acetic acid/30% propan-2-ol, 2% Triton X-100, 2% octylglucoside or 2% SDS.

2.3. *SDS-polyacrylamide gel electrophoresis*

Samples of chromaffin granule lysate or membranes were subjected to electrophoresis as in [15] with a stacking gel containing 4% acrylamide and a running gel containing 13.5% acrylamide and 5 M urea.

2.4. *Electrophoretic transfer of proteins to nitrocellulose and immunochemical detection*

Proteins were electrophoretically transferred to nitrocellulose paper (0.22 μm, Schleicher and Schüll) in 25 mM Tris/192 mM glycine/20% methanol, containing 0.1% SDS [16] at 5 V/cm using a transblot cell (Biorad).

Prior to immunochemical detection free protein-binding sites on the nitrocellulose paper were blocked by incubation with 5% bovine serum albumin in TBS (20 mM Tris-HCl, 0.5 M NaCl, pH 7.4) for 1 h. The paper was then incubated with a 1:200 dilution of anti-proenkephalin(95–117) serum, followed by a 1:200 dilution of goat anti-rabbit IgG (Calbiochem) and finally a 1:1000 dilution of peroxidase–anti-peroxidase complex (Cappel Laboratories, PA). All antibody dilutions were prepared in TBS/1% bovine serum albumin/0.05% Tween 20 and incubations were for 1 h. The nitrocellulose was washed between antibody incubations briefly with distilled water and subsequently with two 10 min washes with TBS/0.05% Tween 20. After the final antibody incubation the nitrocellulose was washed in TBS and peroxidase activity visualized using 0.2 mg/ml diaminobenzidine/0.005% H_2O_2 in TBS.

2.5. *Detection of enkephalin-containing proteins*

To characterize further proenkephalin-like material detected by immunoblotting lanes of nitrocellulose were cut into 2.5-mm slices. The nitrocellulose pieces were subjected to sequential digestion with trypsin (TPCK-treated, Sigma) followed by carboxypeptidase B (Sigma) as described in [7]. Met-enkephalin released by this treatment was determined by radioimmunoassay according to [12]. The Met-enkephalin antiserum is predominantly directed to the C-terminus of the peptide [17] and was a generous gift from Dr Steven L. Sabol, National Institutes of Health.

3. RESULTS

A comparison of proenkephalin-like material visualized by immunoblotting with anti-proenkephalin(95–117) serum in both lysate and membranes from bovine chromaffin granules is shown in fig.1. A range of molecular mass species of proenkephalin-like material was detected in chromaffin granule lysates. The major bands corresponded to proteins of 21.5 and 15.5 kDa, while other species corresponding to 27 kDa and smaller molecular masses were also detected. By comparison, the major form in chromaffin granule membranes had a molecular mass of 27 kDa with minor bands corresponding to proteins of 20.5 and 15.5 kDa. No bands could be detected in either lysates or membranes in control experiments using

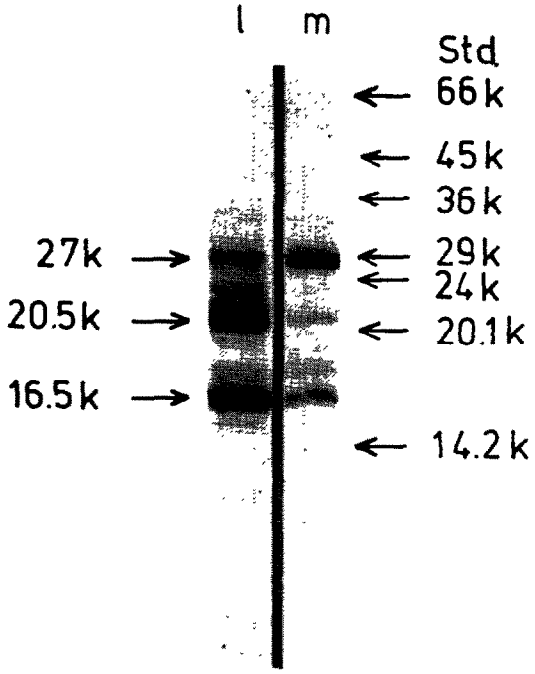

Fig.1. Immunoblotting of bovine adrenal chromaffin granule membranes and lysate. Samples of chromaffin granule lysate (l) and membranes (m) were subjected to electrophoresis and the proteins transferred to nitrocellulose. Immunological detection utilized a primary antiserum which was specific for determinants within the 95–117 sequence of bovine proenkephalin. Molecular mass marker proteins were bovine serum albumin (66 kDa), ovalbumin (44 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20.1 kDa) and lactalbumin (14 kDa).

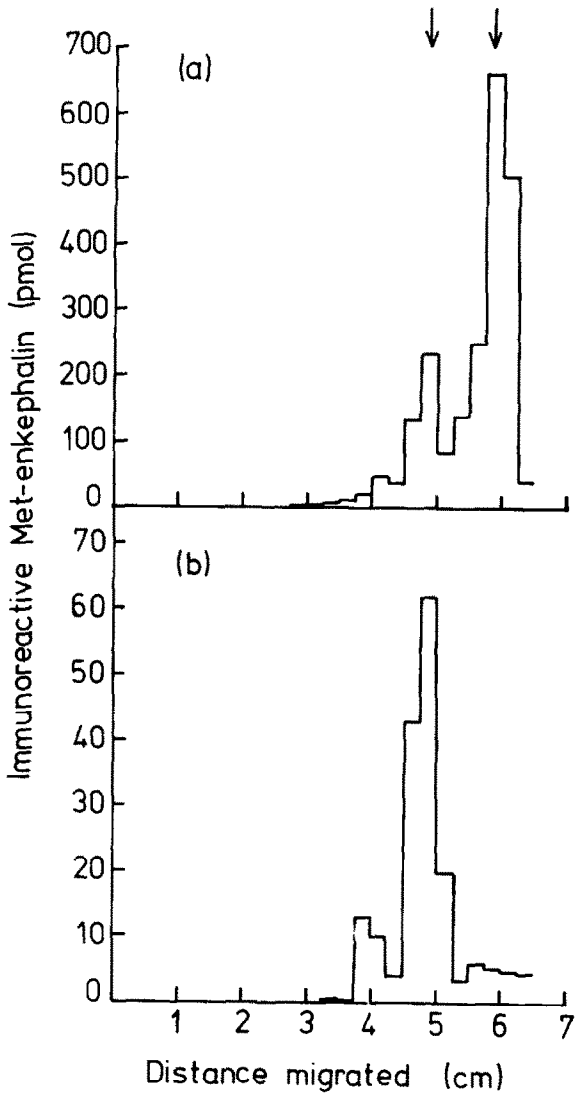

Fig.2. Comparison of the amount of enkephalin released from digestion of immunoblots of proenkephalin-like material from bovine adrenal chromaffin granule membranes and lysates. Samples of chromaffin granule lysate (a) and membranes (b) were subjected to electrophoresis and transferred to nitrocellulose. The nitrocellulose strips were cut into 2.5-mm slices and digested sequentially with trypsin followed by carboxypeptidase B. Met-enkephalin immunoreactivity generated was quantified by radioimmunoassay. The results are expressed in terms of the total immunoreactivity generated from membrane and soluble components derived from the same amount of intact chromaffin granules. The arrows indicate the positions corresponding to 27- and 20-kDa forms of proenkephalin-like material.

antiserum which had been preadsorbed with synthetic proenkephalin(95–117).

We were interested to find a high molecular mass species of proenkephalin (27 kDa) associated with chromaffin granule membranes. Further experiments were directed at determining the proportion of total 27-kDa proenkephalin that was associated with membranes fractions and the nature of the association of this precursor with the membranes. The total enkephalin content of both the 27- and 20-kDa forms present in both membranes and lysate was determined after trypsin and carboxypeptidase B digestion of these bands (fig.2). In both cases, bands detected with anti-proenkephalin(95–117) serum gave peaks of

enkephalin immunoreactivity following enzymic digestion (fig.1). These data indicate that 22% of the total 27-kDa material is associated with the chromaffin granule membranes whereas 99% of the 21.5-kDa material is present in chromaffin granule lysates.

Chromaffin granule membranes were extracted under different conditions and the amount of 27-kDa proenkephalin material solubilized quantified by radioimmunoassay. Immunoreactivity remaining associated with chromaffin granule membranes after various treatments was quantified by radioimmunoassay after solubilization of membrane pellets with 2% SDS. The results of these experiments are shown in table 1. Treatment of washed chromaffin granule membranes with 1.0 M KCl extracted some 26% of the total immunoreactive material which was only slightly greater than that extracted by buffer alone. Extraction of the majority of proenkephalin material required high concentrations of chaotropic agents or detergents. A mixture of 1 M acetic acid/30% propan-2-ol was almost as effective at extracting proenkephalin as sodium thiocyanate. Non-ionic detergents were also effective at extracting membrane-associated material. In all cases both residual and extracted immunoreactivity were also analysed by SDS-polyacrylamide gel electrophoresis and immunoblotting (fig.3). Regardless of the type of extraction the major form of proenkephalin-like material had a molecular mass of 27 kDa.

Table 1

Extraction of immunoreactive proenkephalin from bovine adrenal chromaffin granule membranes

Extraction conditions	Immunoreactive proenkephalin (ng)		Extraction (%)
	Supernatant	Pellet	
Phosphate buffer	174.3	703.5	19.9
1.0 M KCl	216.2	602.3	26.4
2.0 M NaSCN	812.5	154.4	84.0
1 M acetic acid	653.3	369.3	63.9
1 M acetic acid/ 30% propanol	708.0	157.0	81.2
2% octylglucoside	880.8	44.1	95.2
2% Triton X-100	862.0	81.2	91.4
2% SDS	1219.0	13.8	98.9

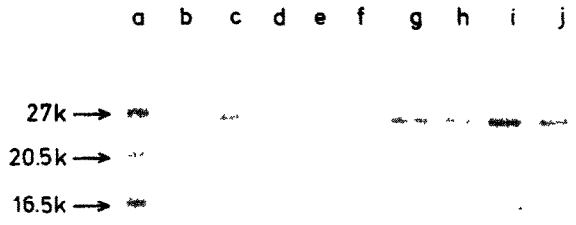

Fig.3. Analysis by immunoblotting of proenkephalin-like material remaining in pellets and extracted after various treatments of washed chromaffin granule membranes. Samples of chromaffin granule membranes were extracted under different conditions and centrifuged at 100000 × *g* for 30 min at 4°C. Aliquots of the resulting pellets and supernatants were subjected to immunoblotting. Pellets (a–e) and supernatants (f–j) were obtained after extracting membranes with phosphate buffer, 2 M NaSCN, 1 M acetic acid/30% propan-2-ol, 2% octyl glucoside and 2% Triton X-100, respectively. The amount of proenkephalin extracted from membranes and remaining in pellets after solubilization with 2% SDS was determined using the proenkephalin(95–117) radioimmunoassay and the results are given in table 1.

4. DISCUSSION

An antiserum which recognized high molecular mass EC proteins has enabled the characterization of intermediates in the processing of proenkephalin in both soluble and membrane fractions from purified chromaffin granules by immunoblotting.

A striking difference was observed in the molecular mass distribution between chromaffin granule membranes and lysates. A high molecular mass (27 kDa) form of proenkephalin predominated in the membrane fraction although this form was also present in the soluble lysate. The soluble and membrane-associated forms were also shown to be identical in a non-urea containing SDS-polyacrylamide gel electrophoresis system (not shown). This system, however, further resolved both membrane and soluble forms into two components differing by approx. 1500 Da.

Quantitative comparison of the enkephalin released from membrane and soluble forms of pro-

enkephalin showed that a significant proportion of the 27-kDa material was membrane-associated while the 20.5-kDa intermediate of proenkephalin was predominantly soluble.

In a previous study, EC proteins in bovine adrenal medulla were characterized by immunoblotting using antisera to Met-enkephalin, synenkephalin and Met-enkephalin-Arg^6-Gly^7-Leu^8 in combination with specific radioimmunoassays [7]. Comparison with this study suggests that the 27-kDa form of proenkephalin corresponds to a 23.3-kDa N-terminal fragment of proenkephalin (residues 1–206) having a Leu-enkephalin sequence at the C-terminus [7]. It now appears that a significant proportion of this material is associated with chromaffin granule membranes. Allowing for repeated freeze-thawing and washing during the preparation of the membranes the proportion of 27-kDa proenkephalin associated with the membrane may be an underestimate.

The 27-kDa material is tightly associated with chromaffin granule membranes by ionic or hydrophobic interactions as treatment with 2 M NaSCN was necessary to extract the majority of it. Non-ionic detergents were very effective at extracting this material although these treatments also solubilized considerable amounts of membrane proteins. Treatment of the membranes with 1 M acetic acid/30% propan-2-ol also extracted a large amount of membrane-associated proenkephalin-like material. The use of volatile components has obvious advantages for the further characterization of the membrane-associated form of proenkephalin. Regardless of the method, the molecular forms of proenkephalin extracted were found to be identical by immunoblotting.

The association of prohormones with secretory granule membranes is a widespread occurrence, possibly involving specific receptors. Proglucagon, prosomatostatin, proinsulin and proopiomelanocortin have been shown to be associated with secretory granule membranes [8,9]. In addition some of these prohormones were shown to bind to secretory granule membranes with a pH optimum consistent with the internal pH of secretory granules [8]. These previous studies found a much higher proportion of the prohormone associated with membranes (~80%) than in this study. The main difference is that the previous work was concerned with newly synthesized, biosynthetically labelled prohormones while we are looking for total proenkephalin-like material in the granules after isolation. The 27-kDa material is also likely to be a high molecular mass intermediate of proenkephalin which may not behave identically to the intact precursor.

The functional significance of the membrane association of prohormones is not clear at present. An attractive possibility is that this may be involved in the targeting of prohormones to secretory granules. It is well established that specific receptors are involved in the targeting of enzymes into lysosomes [18–20]. Binding to membranes may also be necessary for the orientation of precursor molecules for correct processing and this is supported by the association of some processing enzymes with membrane fractions [10,11]. It is not clear at present whether membrane association of proenkephalin is important to processing although membrane-associated proenkephalin is certainly susceptible to cleavage by exogenous proteases such as trypsin (Birch and Christie, unpublished).

It seems likely that membrane association is an important event in the biosynthesis of hormones and neuropeptides. Clarification will require the isolation of membrane components involved in binding and full characterization of the membrane-associated precursors and specific processing enzymes.

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