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SOMATOSTATIN PRECURSORS : EVIDENCE FOR PRESENCE IN AND RELEASE FROM RAT MEDIAN EMINENCE AND NEUROHYPOPHYSIS

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SUMMARY: Characterization of somatostatin-like immunoreactivity in rat median eminence and neurohypophysis by gel chromatography yielded two high molecular weight forms in addition to the tetradecapeptide somatostatin. The two larger molecules comprised 5% and 35% of the total tissue immunoreactivity, showed molecular weights of 25000 and 4000 dalton and were both releaseable in vitro in response to depolarizing stimuli. Their characteristic elution volumes remained unchanged after treatment with dithiothreitol or boiling in 8 M urea. Gentle trypsinization of the 25000 dalton molecule resulted in partial conversion into immunoreactive material coeluting with the tetradecapeptide somatostatin. Since the antibody employed in these studies is specific for the central and C-terminal portions of the tetradecapeptide somatostatin the present data suggest that both high molecular weight forms represent N-terminal extensions of somatostatin and that the 25000 molecular weight material might represent a prohormone for somatostatin.

INTRODUCTION

It is now well established that the biosynthesis of secretory peptides involves ribosomal synthesis of high molecular weight precursor forms which are subsequently processed by specific enzymatic cleavage (1,2). Although a number of reports have suggested the presence in different tissues of somatostatin-like immunoreactivity (SLI) with molecular weight greater than that of the tetradecapeptide somatostatin (SRIF), a precursor molecule for SRIF has not yet been identified with certainty (3 - 6).

The present study was undertaken to characterize SLI in the rat median eminence and neurohypophysis, tissues previously shown to contain a high

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Abbreviations: SLI, somatostatin-like immunoreactivity; SRIF, tetradecapeptide somatostatin; DTT, dithiothreitol; 25 K, 25000 dalton; 4 K, 4000 dalton.

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concentration of somatostatin (7,8). We report here that two high molecular weight forms of SLI are present in the hypothalamus, that both forms are released acutely in response to depolarizing stimuli and that at least one of them may represent a precursor (prosomatostatin) molecule.

MATERIALS AND METHODS

Tissue Extracts. Adult male Sprague-Dawley rats (150-250 g) were used in the experiments. The animals were kept in a temperature and humidity controlled room and were fed laboratory chow and tap water ad libitum. They were killed by instant decapitation and the median eminence and neurohypophysis were removed as separate fragments and placed in 1 ml 1 N acetic acid at 0°C. The tissues were extracted by sonication (Branson sonifier, model 350), the homogenate heated for 5 min in a boiling water bath, rapidly chilled and centrifuged for 10 min at 900 x g (3). The supernatant was then analysed by gel chromatography.

Gel filtration and radioimmunoassay of somatostatin. Gel filtration was performed on Sephadex G-50 superfine and Sephadex G-100 columns, developped in 6 M urea - 0.05 M phosphate buffer (pH 7.5) or 2 N acetic acid (pH 2.3). The content of immunoreactive somatostatin in each fraction was determined by radioimmunoassay using an antibody directed against the central and C-terminal portions of the SRIF molecule (9). Non-specific interference by urea and acid in the radioimmunoassay was prevented by dilution of the samples prior to assay. The following markers were used to calibrate the columns: Dextran blue, bovine serum albumin, ovalbumin, ribonuclease A, chymotrypsinogen A (Pharmacia, Fine Chemicals, Piscataway, N.J., USA); cytochrome C (Boehringer Mannheim, FRG); [^{125}I] porcine insulin (Novo Industries, Bagsvaerd, Denmark) (10); [^{125}I] pancreatic polypeptide (11); SRIF (Ayerst, Montreal, Canada); ^{125}I (New England Nuclear, Boston, USA). Elution volumes were expressed as partition coefficients K_D where $K_D = (V_e-V_o)/(V_s-V_o)$; V_e, elution volume; V_o, void volume; V_s, salt volume.

Dithiothreitol and trypsin experiments. Column fractions eluted in 2 N acetic acid containing higher molecular forms of SLI were pooled separately, lyophilized, reconstituted in 0.05 PO_4 buffer (pH 7.5) and used for dithiothreitol (DTT) and trypsin experiments. Treatment with DTT was performed by exposing SLI to DTT (100-fold molar excess) at 50°C under 100% N_2 for 3 hr (10). Trypsinization was carried out by treatment of SLI with trypsin (TPCK-trypsin, Worthington Biochemical Corp., Freehold N.J.) at a concentration of 0.1% or 5% w/w at 25°C, pH 8.2 for 1, 10 or 100 min. The reaction was terminated by addition of 1 ug soyabean trypsin inhibitor (Boehringer Mannheim, FRG).

Somastatin release experiments. Pools of 5 median eminences or neurohypophysis were incubated for 30 min periods in 1 ml Locke's solution as previously described (7,8). Somatostatin release was stimulated by increasing the K^+ concentration in the medium from 5.6 to 56 mM. Medium containing the released somatostatin was chromatographed on Sephadex G 50 columns as described above for the extracts.

Statistical analysis. To check for paralellism between serial dilution slopes of unknown substances and SRIF, the residual variances of the two curves were tested for homogeneity by a f-test and the lines were tested for parallelism by a t-test both with the aid of a computer program (13). Significant non-parallelism was indicated by a p-value < 0.05 in the t-test.

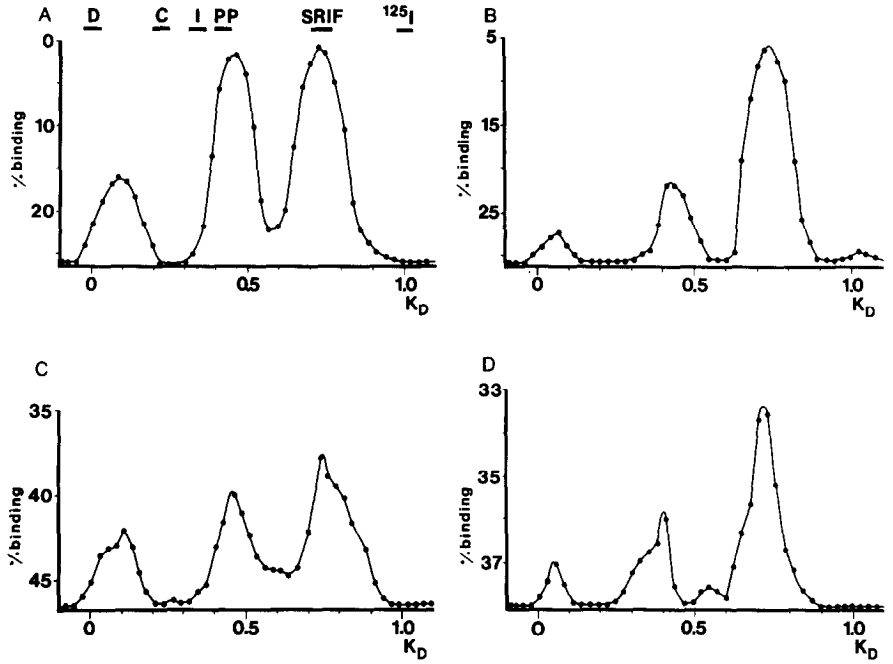

Fig. 1. Characterization of immunoreactive somatostatin in tissue extracts and incubation media by Sephadex G-50 (superfine) gel chromatography. A 1.15x56cm column was eluted with 6 M urea in 0.05 M PO_4 buffer, pH 7.5. K_D = partition coefficient. Molecular weight markers: D, dextran blue; C, cytochrome C; I, [125I]-insulin; PP, [125I]-pancreatic polypeptide; SRIF, synthetic somatostatin (tetradecapeptide); ^{125}I, radioactive iodine.

A : Extract of neurohypophysis. B : Extract of median eminence.
C : Incubation medium containing immunoreactive somatostatin released in vitro from neurohypophysis following stimulation by K^+ (56 mM).
D : Incubation medium containing immunoreactive somatostatin released in vitro from median eminence following stimulation by K^+ (56 mM).

RESULTS

Sephadex G-50 filtration of extracts of both median eminence and neurohypophysis showed three peaks of immunoreactive somatostatin (fig. 1A and B). Identical elution patterns were obtained with columns developed in either 6 M urea or 2 N acetic acid. 60% of the total immunoreactivity coeluted with synthetic SRIF at K_D 0.73. The additional peaks comprised higher molecular weight material eluting with K_D of 0.11 (peak I SLI) and 0.43 (peak II SLI). SLI in peak I accounted for approximately 5% of the total immunoreactivity whereas peak II was more prominent and represented about 35% of the total immunoreactivity. Estimation of the molecular weights of the two peaks using a Sephadex G-100 column calibrated with markers of known molecular weight

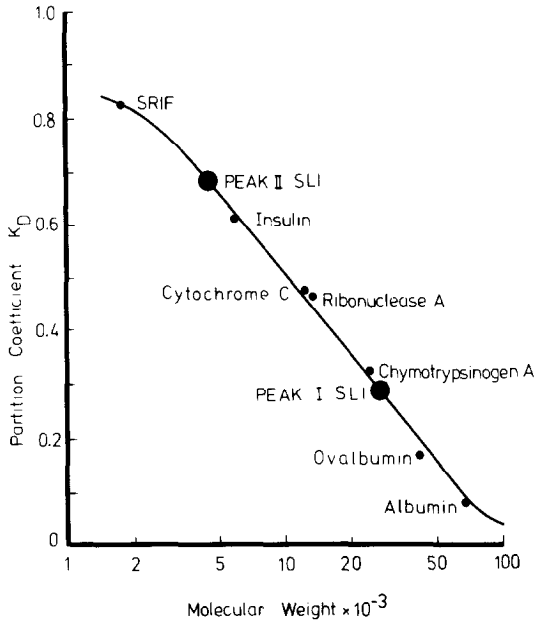

Fig. 2. Molecular weight calibration curve using Sephadex G-100. (0.9 x 58 cm column; flow rate 0.15 ml/min; hydrostatic pressure, 100 cmH_2O; eluting buffer, 6 M urea in 0.05 M PO_4 buffer, pH 7.5).

showed apparent molecular weights of 25000 dalton and 4000 dalton for peak I and II SLI respectively (fig 2).

The in vitro release of immunoreactive somatostatin from neurohypophysis and median eminence fragments was increased 9 fold and 5 fold respectively over basal levels in response to high K^+ (56 mM) stimulation. The release was specific and was dependent on the presence of Ca^{++} in the incubation medium. Gel filtration of the released immunoreactivity yielded 3 peaks (fig 1C and D) with K_D values identical to those obtained for the tissue extracts.

In order to further characterize the higher molecular weight materials, serial dilutions of peak I and peak II SLI were assayed. The resulting inhibiton curves showed parallelism with synthetic SRIF (fig 3). DTT treatment of either of the two forms of 'big' SLI did not yield any immunoreactive material corresponding to SRIF. Also boiling in 8 M urea for 5 min did not alter the elution position of either peak I or peak II SLI. Incubation of peak I and peak II SLI with trypsin 5% (w/w) for 10 min led to a complete loss of

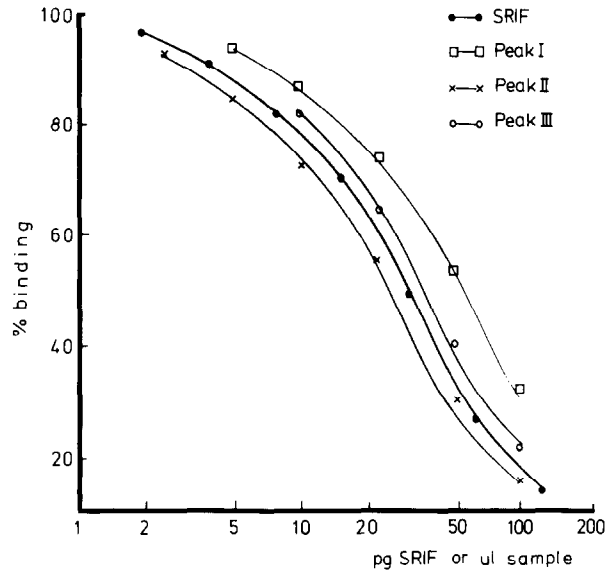

Fig. 3. Parallelism between serial dilution slopes of peak I, II and III SLI with synthetic SRIF. (Peak III: material coeluting with SRIF).

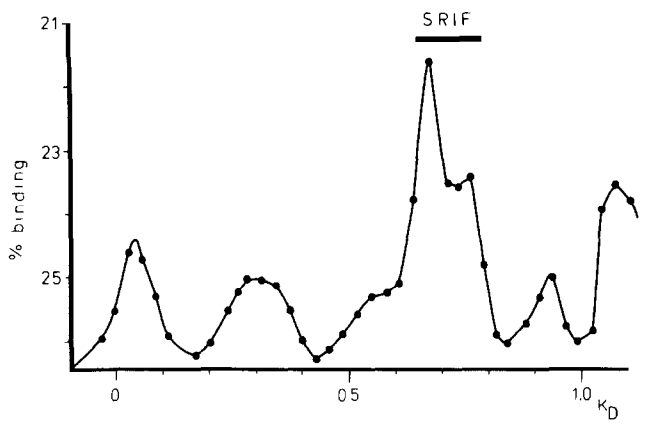

Fig. 4. Elution profile of peak I SLI after gentle trypsinization (0.1%(w/w) 100 min) on Sephadex G-50 (superfine). Elution buffer 6M urea in 0.05 M PO_4 buffer, pH 7.5)

immunoreactivity. Using more gentle conditions however (trypsin 0.1% (w/w) for 100 min), the bulk of peak I SLI was converted to a lower molecular weight immunoreactive species coeluting with SRIF (fig 4). Two additional peaks of immunoreactivity with K_D 0.30 and 0.81 were also obtained. These probably represent an intermediate breakdown product of peak I SLI and a fragment of the tetradecapeptide SRIF respectively. Treatment of peak II SLI with the

low concentration of trypsin led to a broad peak of immunoreactivity (K_D 0.47-1.0) which overlapped the elution volume of SRIF (K_D 0.73) suggesting the presence of both SRIF and other immunoreactive fragments.

DISCUSSION

The existence of high molecular weight forms of SLI in the hypothalamus has been previously demonstrated by a number of investigators (3-6). In the present study we have further characterized this 'big' SLI in median eminence and neurohypophysial extracts and shown it to consist of two polypeptides with molecular weights in the region of 25 K and 4 K which together account for approximately 40% of total immunoreactivity. Furthermore the in vitro incubation studies revealed that both high molecular weight forms are released from these tissues in response to a depolarizing stimulus, findings comparable to those reported in non-neural cells (1,2,14).

Since our gel filtration studies were performed under strongly denaturating conditions (6 M urea or 2 N acetic acid) and since both molecules remained essentially intact even after treatment with DTT or boiling 8 M urea, it is unlikely that they represent protein-bound, aggregated or polymerized SRIF. Because trypsin-like enzymes have been implicated in the post-translational modification of peptide hormone precursors (1,2), the present finding of conversion of the 25 K SLI peptide to material coeluting with SRIF by gentle trypsinization provides evidence for a role of the 25 K molecule as a prohormone for SRIF. Since the specificity of the antibody employed in these studies is directed toward the middle and C-terminal regions of SRIF it would appear that the 25 K molecule is a N-terminal extension of SRIF connected to the tetradecapeptide via basic amino acid residues. The relationship of the 4 K molecule to SRIF is less clear from the present studies. It too may represent a N-terminal extension of SRIF although its role as a precursor of SRIF is uncertain because of the inconclusive results obtained with trypsinization.

Further studies of the biosynthesis of SRIF by hypothalamic neurons coupled with structural analysis of the high molecular weight forms should help to clarify the role of these molecules as precursors for SRIF and possibly other hypothalamic peptides.

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