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PROCESSING OF SOMATOSTATIN PRECURSORS: EVIDENCE FOR ENZYMATIC CLEAVAGE BY HYPOTHALAMIC EXTRACT

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SUMMARY: The action of enzymes extracted from rat hypothalamus on the previously characterized high molecular weight forms of hypothalamic somatostatin-like immunoreactivity (4 K SLI and 25 K SLI) has been investigated in vitro in order to further define the role of these molecules as possible precursors for tetradecapeptide somatostatin (SRIF). Studies of the degradation of endogenous SLI and of synthetic SRIF by hypothalamic enzymes showed that the time course of breakdown of endogenous SLI is markedly slower than that of synthetic SRIF due to the relative stability of 25 K SLI as well as the generation of at least two new immunoreactive molecules. Incubation of purified 25 K SLI with SLI-free hypothalamic extract showed after 10 to 30 min newly formed immunoreactive material of an intermediate size between 25 K SLI and 4 K SLI and after 60 min the emergence of material coeluting with SRIF. These data show that the hypothalamus contains the enzymes necessary for degrading endogenous SLI and for processing the 25 K SLI molecule to SRIF providing further evidence that 25 K SLI might be a biosynthetic precursor for SRIF.

INTRODUCTION

There is now considerable evidence to suggest that the biosynthesis of secretory peptides occurs via ribosomal synthesis of high molecular weight precursor forms which are further processed by enzymatic cleavage. Enzymes capable of generating a peptide from its precursor molecule have been demonstrated in several secretory systems (1-4). Although the biosynthesis of somatostatin has been clearly established in the pancreas (5), little is currently known about its synthesis in nerve cells.

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Abbreviations: SLI, somatostatin-like immunoreactivity; SRIF, tetradecapeptide somatostatin; 25 K, 25000 dalton; 4 K, 4000 dalton; 1.6 K, 1600 dalton; K_D, partition coefficient.

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We have recently demonstrated that somatostatin-like immunoreactivity (SLI) in rat hypothalamus is composed of two high m.w. forms (apparent m.w. of 4 K and 25 K) in addition to tetradecapeptide somatostatin (SRIF, m.w. 1.6 K) and that the 25 K SLI molecule can be converted to SRIF by gentle trypsinization (6). In the present study we have investigated the effect of hypothalamic enzymes on the processing of 25 K SLI. We present evidence that hypothalamic extracts contain the enzymes necessary for generating SRIF from this precursor molecule.

MATERIALS AND METHODS

Hypothalamic Extracts. Hypothalami were obtained from male Sprague-Dawley rats sacrificed by rapid decapitation and the tissue homogenized by a gentle procedure to preserve enzymatic activity. This included sonication for 1 min in 0.1 M PO_4 buffer pH 7.4 at 0 C followed by centrifugation at 1000 x g for 10 min to remove the insoluble material. The supernatant containing endogenous SLI and enzymes was kept for subsequent studies.

Gel Chromatography and RIA of SLI. Gel filtration was performed on Sephadex G-50 columns (1.15 x 56 cm) equilibrated either with 6 M urea in 0.05 M PO_4 buffer (pH 7.5) or 2 N acetic acid. The SLI content of individual fractions was determined by RIA using an antibody with specificity directed toward the central portion of tetradecapeptide SRIF (7). Elution volumes were expressed as partition coefficient 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.

Preparation of Purified SLI and SLI-free Hypothalamic Extracts. Endogenous SLI was removed from hypothalamic extracts by passage through an immunoaffinity column prepared by conjugation of polyethylene glycol precipitated sheep anti SRIF globulin to Affigel 10 (Biorad Products) (8). The void volume fractions of the column containing the endogenous enzymes were pooled and lyophilized. Non-specifically bound protein was eluted from the column with 1 N NaCl, following which specifically bound SLI was eluted with 1 N acetic acid and rechromatographed on Sephadex G-50 columns developed in 2 N acetic acid. Fractions containing purified 25 K SLI were pooled and lyophilized.

Spontaneous Degradation of SLI in Hypothalamic Extracts. The spontaneous degradation of endogenous SLI was studied by incubating hypothalamic extracts at 37 C, pH 7.4. Aliquots were removed at various time intervals, acidified (pH 2.5) with glacial acetic acid, boiled for 5 min to inactivate enzymes and the total SLI content determined by RIA. In addition, the SLI components in the extracts at 0 hr, 1 hr and 24 hr were analysed by gel chromatography. Degradation of SRIF was studied by incubating 0.4 ug synthetic SRIF (Ayerst, Montreal) with SLI-free hypothalamic extracts at 37 C, pH 7.4. Aliquots of the mixture were removed and the SLI content determined by RIA. Control experiments were performed using heat inactivated extracts.

Incubation of 25 K SLI With SLI-free Hypothalamic Extracts. Purified 25 K SLI was incubated with SLI-free hypothalamic extract at 37 C, pH 7.4. Aliquots were taken at 0, 10, 30 and 60 min, acidified to pH 2.5, boiled and reanalysed by gel chromatography to determine the various immunoreactive components.

RESULTS

The time course of degradation of endogenous SLI in hypothalamic extracts is shown in Fig. 1. There was a 42% loss of immunoreactivity by 15 min, 77%

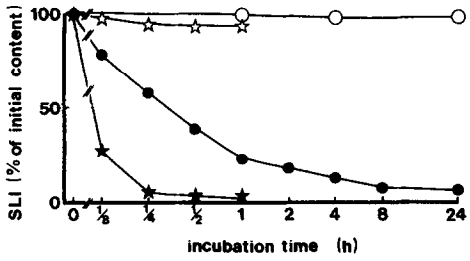

Fig. 1. Time course of in vitro degradation of endogenous SLI (●), and of synthetic SRIF (★) during incubation with hypothalamic extracts. Control experiments included incubation of endogenous SLI (O) and of synthetic SRIF (☆) in heat inactivated hypothalamic extracts. Each point represents the mean of a duplicate determination.

by 1 hr and 94% by 24 hr. By contrast synthetic SRIF was degraded more rapidly with 51% loss of activity by 4 min, and 95% loss by 15 min.

Chromatographic analysis of endogenous SLI in the hypothalamic extracts revealed at time 0 min, 3 peaks of SLI corresponding to 25 K SLI, 4 K SLI and tetradecapeptide SRIF (Fig. 2). The profile at 1 hr showed considerable loss of 4 K SLI and of material coeluting with SRIF. At 24 hours both these peaks were completely absent. By contrast 25 K SLI persisted in significant amounts even after 24 hours suggesting that this material is relatively more stable than 4 K SLI or SRIF.

In addition to the three peaks of immunoreactivity observed at time 0, two other SLI peaks were demonstrated during the course of the degradation experi-

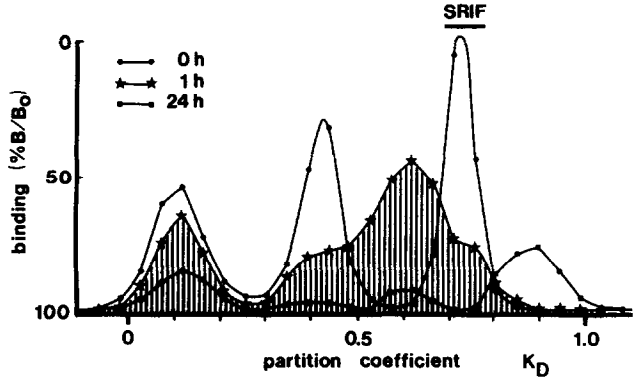

Fig. 2. Characterization of immunoreactive somatostatin in hypothalamic extracts after 0, 1 and 24 hours of incubation at 37 C, pH 7.4. A Sephadex G-50 column equilibrated with 6 M urea in 0.05 M PO_4 buffer pH 7.5 was used.

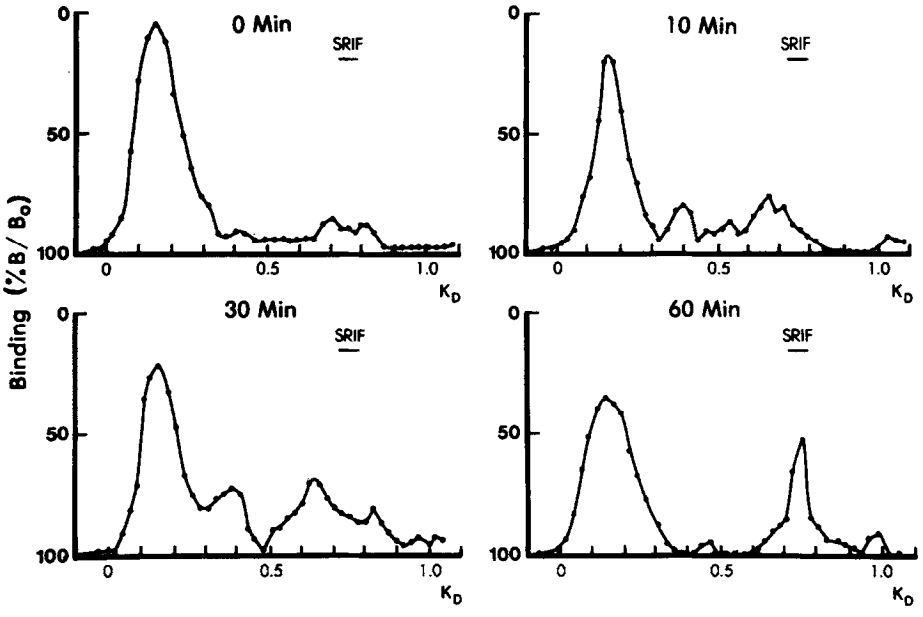

Fig. 3. Cleavage of 25 K SLI by hypothalamic enzymes. 25 K SLI material was incubated with SLI-free hypothalamic extracts and the immunoreactive material in aliquots taken after 0, 10, 30 and 60 min characterized by gel chromatography. K_D = partition coefficient. ——: elution position of tetradecapeptide SRIF (K_D = 0.73).

ment. One of these (K_D = 0.6) was intermediate in size between 4 K SLI and SRIF and was the predominant immunoreactive form at 1 hr whereas the other (K_D = 0.9) eluted after SRIF as a prominent peak at 24 hr and probably represents a stable fragment.

When purified 25 K SLI was incubated with hypothalamic extracts from which endogenous SLI had been removed by immunoabsorption, rechromatography of the aliquots taken from the incubation mixture at different time intervals showed after 10 min the emergence of newly formed immunoreactive material eluting with K_D values of 0.4 and 0.6 (Fig. 3). At 60 min a sharp peak appeared in the elution position of SRIF whereas the intermediate peaks disappeared (Fig. 3).

DISCUSSION

Of the two high m.w. forms of SLI previously identified in the rat hypothalamus, one of these (25 K SLI) was shown to yield SRIF on partial tryptic digestion. In the present study we have investigated the action of endogenous

hypothalamic enzymes on the high m.w. forms of SLI in an attempt to further delineate their role as possible precursors for SRIF.

Incubation of hypothalamic extracts at neutral pH showed that the total immunoreactivity is rapidly degraded. The enzymatic nature of this process is indicated by its sensitivity to heating. The time course of inactivation of endogenous SLI (comprising high m.w. SLI as well as material corresponding to SRIF), however, was much slower than that of synthetic SRIF. This was due to the relatively greater stability of 25 K SLI compared to SRIF as well as to the generation of at least 2 new immunoreactive molecules during incubation. Studies of the enzymatic degradation of synthetic SRIF by hypothalamic extracts have shown that the peptide is capable of being cleaved by both aminopeptidases and endopeptidases (9-11). Whereas N-terminal modification of SRIF would preserve immunoreactivity, cleavage by endopeptidases (Trp-Lys- bond at position 8-9 of SRIF molecule) would result in fragments not recognizable by the antibody used in the present study (7).

Our studies of the action of SLI-free hypothalamic extracts on purified 25 K SLI suggest that hypothalamic tissue contains the enzymes necessary for converting this molecule to tetradecapeptide SRIF. Since this process can be mimicked by trypsin (6, 12) it would appear that the cleavage specificity of the hypothalamic enzymatic activity resembles that of trypsin, a similarity that has also been observed for other enzyme systems involved in the processing of biosynthetic precursors (1-4). A high m.w. form of SLI has recently been identified in the mouse hypothalamus which is capable of being processed in vitro by hypothalamic extracts into SRIF (13). This molecule, however, has an apparent m.w. of 15,000 and can not be converted to SRIF by trypsin suggesting that it differs considerably from the 25 K SLI in rat hypothalamus.

The precise nature of 4 K SLI remains uncertain. In our present study of the degradation of 25 K SLI, one of the intermediate forms arising after 10 to 30 min incubation eluted in a position corresponding to 4 K SLI (K_D = 0.4). This would suggest that 4 K SLI represents an intermediate in the sequential

breakdown process of the precursor molecule. Pradayrol et al have recently isolated and characterized a high m.w. form of SLI in porcine intestine (14). This 28 amino acid molecule represents an N-terminal extension of SRIF connected to the tetradecapeptide sequence via two basic amino acids and might thus be a biosynthetic precursor for SRIF. The correspondence of this molecule to the 4 K SLI identified in our study in the rat hypothalamus remains to be determined. Further studies of the biosynthesis of somatostatin-like peptides combined with their structural analysis will be required to clarify this question.

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