In vivo synthesis and processing of rat hypothalamic prosomatostatin

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The in vivo incorporation of [^{3}H]phenylalanine into an apparent 15 kDa prosomatostatin was observed in the hypothalamus of rats injected with the labeled amino acid in the third ventricle. Precursor-product relationships were established between this newly synthesized material and both somatostatin-28 and -14.

Prosomatostatin *Hypothalamus* *Pulse-chase*

1. INTRODUCTION

Somatostatin (somatostatin-14) is a tetradecapeptide hormone present in both neural and gastrointestinal tracts [1–3]. In the central nervous system, it is distributed throughout various brain areas [4] together with another form: somatostatin-28 [5]. Somatostatin-28, first discovered in both porcine intestine [6] and hypothalamus [7] and in ovine hypothalamus [8,9], corresponds to an NH_2-terminally extended somatostatin-14 containing 28 amino acids.

Hypothalamus, where somatostatin-positive cell bodies are clustered within the periventricular nuclei [10,11], has been extensively used to study the neural biosynthesis of both somatostatin-14 and -28. An immunoreactive material with an apparent $M_r = 15\,000$ was identified in mouse [12] and rat [13] hypothalamic extracts. In contrast with the situation encountered in the pancreas [14,15] this material consists of a single, predominant, form which was converted into both somatostatin-14 and -28 by incubation with hypothalamic enzymes [16]. A preprosomatostatin was identified by translating, in a cell-free system, either rodent [17] or bovine [18] hypothalamic mRNAs. The presence of microsomal membranes during the translation yielded a proprotein with a $M_r \sim 15\,000$ [18]. Hypothalamic neurones kept in culture incorporated labelled amino acids into an immunoreactive form ($M_r \sim 15\,000$) which decreased during a chase period while somatostatin-14 and -28 appeared [19]. All these results suggest strongly that both hypothalamic somatostatin-14 and -28 are synthesized via a common precursor with $M_r \sim 15\,000$. Nevertheless, up to now, no in vivo precursor-product relationship between a newly synthesized form and somatostatin-14 and -28 has been reported. Therefore, we have attempted to establish this by performing pulse-chase experiments in the rat. The results we have obtained indicate that an immunoreactive form with $M_r \sim 15\,000$ was detected and that it behaves as a biosynthetic precursor.

Abbreviations: SDS-PAGE, SDS-polyacrylamide gel electrophoresis; HPLC, high-pressure liquid chromatography; TFA, trifluoroacetic acid

2. MATERIALS AND METHODS

Male Wistar rats (260 g, Iffa-Credo, France) were anesthesized with sodium pentobarbital (45–60 mg/kg, i.p., Clin-Midy, France). 200 μCi L-[2,3,4,5,6-^{3}H]phenylalanine (130 Ci/mmol, Amersham, England), concentrated by lyophilisation then dissolved in 2 μl of 150 mM NaCl, were in-

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jected into the third ventricle of each animal using a stereotaxic device (David Kopf Instruments, USA) and according to [20].

After the chase period, the rats were decapitated, the brains were removed then the whole hypothalami were dissected as in [21] and homogenized in 0.1 N HCl containing 8 M·urea, 4 μg/ml aprotinin, 1 μg/ml of pepstatin and 5 mM phenylmethylsulfonyl fluoride. After extraction (16 h) the homogenate was centrifuged at 100 000 × g for 60 min in a Beckman L5-50 ultracentrifuge. The supernate was fractionated on a Sephadex G-50 column (1.2 × 100 cm) equilibrated with 0.1 N formic acid and precalibrated according to [5].

Eluate containing proteins with M_r 10 000–25 000 was pooled and somatostatin-like species were isolated by immunoprecipitation. Antisomatostatin antiserum 36-36 (a generous gift of C. Rougeot, URIA, Institut Pasteur, Paris) was used at a final dilution of 1:6 after IgG chromatographic separation on DEAE-trisacryl. Finally, immunoprecipitated proteins were analyzed by SDS-PAGE. Immunoprecipitation and SDS-PAGE were carried out as in [22].

Fractions from the filtration on Sephadex G-50 which contained together peptides with $M_r < 10\,000$ and low M_r molecules such as urea and [^{3}H]phenylalanine were pooled and passed through a Sep-Pak C18 cartridge (Water Associates, Milford, MA). By washing with 0.1% TFA, urea and [^{3}H]phenylalanine were removed and then the adsorbed peptides were eluted with 80% acetonitrile in 0.1% TFA. After elimination of acetonitrile and TFA by evaporation in a savant Speed Vac concentrator, the somatostatin-like peptides were isolated by immunoaffinity chromatography as described [12], then identified by HPLC using a Beckman system 421. Analyses were performed on a μBondapak C18 column (3.9 × 300 mm) with 25% acetonitrile in 100 mM triethylammonium phosphate (pH 3) as solvent at 1 ml/min. Somatostatin-14 and -28 obtained through CRB (Cambridge, England) were used as standards.

Protein concentration was determined according to Lowry et al. [23].

3. RESULTS

Based on available data in the literature the hypothalamic precursor of both somatostatin-14 and -28 should be expected as a protein with $M_r \sim 15\,000$. Therefore, during a pulse-chase experiment, this protein should be detected early then its disappearance should be concomitant with increase in both somatostatin-14 and -28.

To perform the in vivo labeling experiment [^{3}H]phenylalanine was injected into rat third ventricle and the protein content of hypothalamic extracts was analyzed at various times thereafter.

Preliminary experiments were carried out with [^{35}S]cysteine then [^{3}H]phenylalanine was used owing to its better incorporation into proteins. The third ventricule was selected as injection site because the somatostatin-producing cell bodies are essentially located in the periventricular area surrounding the third ventricule [10,11]. After the chase period, the extract was prepared from the whole hypothalami in order to detect both the precursor produced by the periventricular pericarya and the final products transported to various hypothalamic areas.

The radioactive proteins and peptides of the extracts were chemically characterized by both SDS-PAGE and reverse-phase HPLC, respectively. They were previously selected by gel filtration and purified by either immunoprecipitation or affinity chromatography. Proteins were recovered as a unique peak corresponding to species with M_r 15 000 ±10%. This peak disappeared when immunoprecipitation was carried out in the presence of somatostatin-14 (fig.1). Peptides were recovered as two peaks identified as somatostatin-14 and -28 as coeluted with synthetic standards (fig.2).

To establish a precursor-product relationship between the protein with $M_r \sim 15\,000$ and somatostatin-14 and -28, the radioactivity of these species was measured as a function of the chase period. 30 min after [^{3}H]phenylalanine injection, a protein with $M_r \sim 15\,000$ was the only one detected. Its radioactivity increased for 1 h then decreased and disappeared at 4 h (fig.3). Somatostatin-14 was detected after 2 h whereas somatostatin-28 appeared later, after 4 h. After 20 h, the radioactivity of each peptide was approximately the same as after 4 h with the somatostatin-28 value lower than that of somatostatin-14. Taken together, these variations in radioactivity incorporated into the protein with $M_r \sim 15\,000$ and somatostatin-14 and -28 are consistent with the early synthesis of a

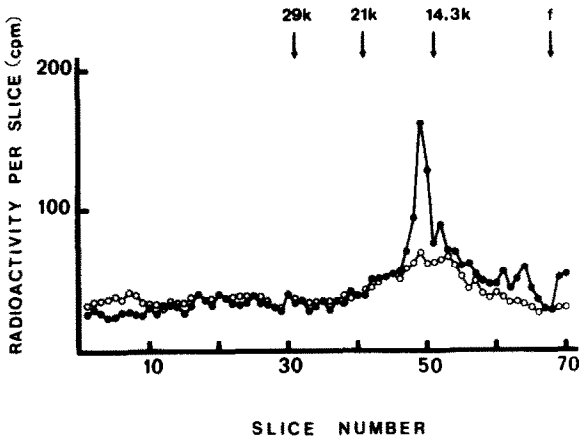

Fig.1. SDS-PAGE of the ^{3}H-labelled somatostatin-like proteins extracted from 5 rat hypothalami 1 h after the injection of [^{3}H]phenylalanine into the third ventricle. The material was immunoprecipitated with antisomatostatin-14 antibodies both in the presence (○) and absence (●) of 10 μg somatostatin-14. Molecular mass markers (in kDa): carbonic anhydrase (29); soybean trypsin inhibitor (21); lysozyme (14.3). f, migration of bromophenol blue.

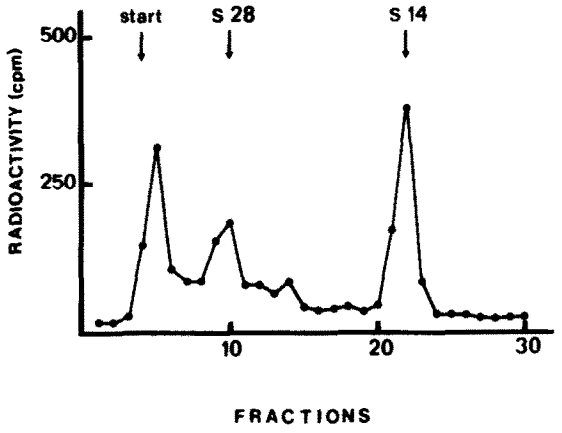

Fig.2. Reverse-phase HPLC of the ^{3}H-labelled somatostatin-like peptides extracted from 5 rat hypothalami 4 h after the injection of [^{3}H]phenylalanine into the third ventricule and purified by immunoaffinity chromatography. Column, Bondapak C18 (3.9×300 mm); isocratic solvent, 25% acetonitrile in 100 mM triethylammonium phosphate, pH 3; flow rate, 1 ml/min; fractions, 1 ml. The elution volumes of both synthetic somatostatin-14 (S-14) and somatostatin-28 (S-28) are indicated by arrows.

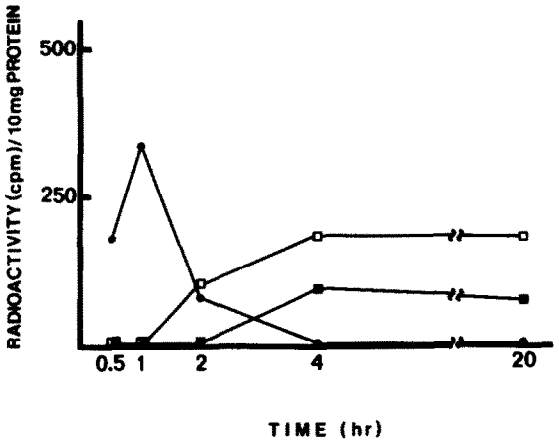

Fig.3. Radioactivity incorporated into prosomatostatin (M_r ~ 15 000) (●), somatostatin-14 (□) and somatostatin-28 (■) at various times after the injection of [^{3}H]phenylalanine into the rat third ventricule. Each plotted value corresponds to the mean radioactivity of 2 or 3 successive experiments carried out with 5 rats each.

precursor molecule and its subsequent processing into final products.

An additional radioactive somatostatin-like peak was occasionally detected on SDS-PAGE after 2, 4 and 20 h, respectively. It was located in the range M_r 8000–10 000. It was not detected after a short period of chase (30 min and 1 h).

4. DISCUSSION

Pulse-chase experiments are suitable for studying the biosynthesis of peptides arising from precursors but some difficulties can be encountered when they are performed in vivo. The presence of unlabelled amino acid in the tissues may have a negative effect on the incorporation by decreasing the radioactivity of the injected amino acid. In tissues where the protein synthesis is multiple, the radioactivity incorporated into each protein can be very low. Although, in our experiments, radioactive somatostatin-like proteins represented only 0.005% of the total radioactive proteins, it was nevertheless possible to isolate and analyze them. In the same way, radioactive somatostatin-14 and -28 (0.5% of the total radioactive peptides) could be purified and characterized. The results obtained show unambiguously that somatostatin-14 and -28 derive from a precursor with M_r 15 000. So, the in

vivo synthesis seems to proceed as indicated by the in vitro experiments [12,13,16–19].

Two points remain unresolved. The first concerns the size of the prosomatostatin. The experimental values correspond to $M_r \sim 15\,000$. On the other hand, Goodman et al. [24] calculated a $M_r = 10\,388$ from the sequence of a rat thyroid carcinoma preprosomatostatin deduced from the cDNA sequence. A post-translational modification could be responsible for this discrepancy. Glycosylation has been evoked but seems unlikely [24]. Another possibility is that prosomatostatin possesses a particular conformation resulting in an anomalous behavior in both SDS-PAGE and gel filtration.

The second point concerns the processing of prosomatostatin into both somatostatin-14 and -28. Somatostatin-28 is generally considered as an intermediate between prosomatostatin and somatostatin-14. A conversion enzyme has been characterized in rat brain extracts which produces in vitro somatostatin-14 from somatostatin-28 [25–27]. On the other hand, it appears that somatostatin-14 can be generated directly from prosomatostatin. Benoit et al. [28] characterized a peptide in rat brain extracts which should correspond to the whole prosomatostatin molecule devoid of the Arg-Lys-somatostatin-14 COOH-terminal fragment. If somatostatin-28 generates somatostatin-14, a precursor-product relationship between the two peptides should be observed during a pulse-chase. In our experiments this was not apparent. Somatostatin-14 was detected at first as if it was produced directly from prosomatostatin. Nevertheless, the alternative possibility that somatostatin-28 could be formed, then immediately, and rapidly converted to somatostatin-14 cannot be excluded. In addition, a low incorporation of radioactivity into somatostatin-28 might remain undetected under our experimental conditions. In contrast, with its late appearance, somatostatin-28 remains present as well as somatostatin-14 after 20 h. This fact, and the absence of a precursor-product relationship between somatostatin-14 and -28, suggest as previously proposed by Van Itallie and Fernstrom [29] that somatostatin-28 is at least in part a 'final' product in the hypothalamus. So, the processing of the prosomatostatin is a complicated phenomenon. Moreover, other peptides, resulting from other cleavages at still unidentified loci can be generated. In our experiments another somatostatin-like peptide was detected after 2 h. According to its apparent M_r it could possibly arise from a cleavage at position 56–57 of the preprosomatostatin sequence [24].

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