SELECTIVE PROCESSING OF THE 15 000 M_r PROSOMATOSTATIN BY MOUSE HYPOTHALAMIC EXTRACTS RELEASES THE TETRADECAPEPTIDE

Alain MOREL, Marc LAUBER and Paul COHEN

Groupe de Neurobiochimie Cellulaire et Moléculaire, Université Pierre et Marie Curie, 96 boulevard Raspail, 75006 Paris, France

Received 19 November 1981

1. Introduction

Brain peptides seem to be synthetized in neuronal systems as larger M_r precursors. A 15 000 M_r form of the tetradecapeptide somatostatin was characterized in hypothalamic extracts [1,2] and its digestion by copurified enzymes led to lower M_r somatostatin-like species [1]. Pre-prosomatostatins with comparable size were identified by cell-free translation of mRNA extracted from the Brockmann organ of the Anglerfish [3,4]. The cloning of cDNA coding for somatostatin precursors from the pancreas was accomplished by 2 groups and indicated some divergence in the sequence of the predicted pre-prohormone [5,6]. These observations were interpreted [5] as suggesting the existence of several (at least 2) genes coding for 2 distinct somatostatin sequences: the normal tetradecapeptide (somatostatin I) and its derivative (Tyr^7–Gly^{10}; somatostatin II). In spite of obvious analogies of molecular size with the pancreatic precursor, little is known about the structure and the function(s) of the somatostatin precursor produced in the central nervous system; in particular, the peptide sequences recognized by the putative converting enzyme(s). Here we demonstrate that hypothalamic extracts contain protease(s) capable of converting selectively the M_r 15 000 precursor into somatostatin I as evidenced by radioimmunological and high-pressure liquid chromatography (HPLC) criteria.

2. Experimental

Phenylmethylsulfonylfluoride (PMSF), trasylol and pepstatin were purchased from Sigma (St Louis MO). Acetronile HPLC grade, was obtained from Prolabo. The synthetic somatostatin was furnished by Clin-Midy (Montpellier) [7], and its purity was checked both by HPLC and mass spectrometry thanks to the Service central d'analyse, CNRS (Vernaison). The antiserum was provided by URIA (Dr Dray) Institut Pasteur (Paris). The analogs (Tyr^{11}) and (Tyr^1)-somatostatin were from Bachem (California).

All experiments were run at 4°C. Mice hypothalami (25 fragments) were removed under a binocular microscope (Nikon type 102) immediately after sacrifice and homogenized in a Potter-Elvejhem (clearance 0.25 μm) in 3 ml 0.1 N HCl, 8 M urea containing 250 kallikrein inhibitor units/ml. Centrifugation was performed for 1 h at 100 000 × g in a SW 50 rotor on a Beckman L5-65 centrifuge. The supernatant solution was submitted to molecular sieve filtration on a Sephadex G-50 column equilibrated and eluted with 0.1 N HCOOH containing 0.5 mM PMSF and 1 mg pepstatin/l. Each fraction was tested by a radioimmunoassay procedure (RIA) using anti-somatostatin 3638 antiserum. The free iodinated tracer was separated from the antibody bound by propanol precipitation after addition of non-immune serum in each fraction. Controls were routinely performed as in [1] to exclude RIA artefacts possibly arising from degradation of the tracer or its sticking to protein components of the samples. Separation and identification of the tetradecapeptide somatostatin were carried out by HPLC on a SP 8000 (Spectra Physics) apparatus using a reverse phase column Lichrosorb 10 RP 18 (250 × 4.6 mm) from Merck. The column was eluted isocratically with a mixture containing 34% (v/v) acetonitrile and 66% (v/v) ammonium acetate 0.05 M, adjusted to pH 4. At the end of the run, a linear gradient of acetonitrile was generated in order to release all fragments possibly with a higher retention

Published by Elsevier/North-Holland Biomedical Press

00145793/81/0000–0000/$02.75

time. Before each run all the collection tubes were coated with bovine serum albumin. After lyophilisation each eluted fraction was tested by RIA.

3. Results and discussion

Chromatography of mice hypothalami extracts on a Sephadex G-50 column separated 3 major immunoreactive peptides with apparent M_r: 15 000, 6000 and 1600. The smaller immunoreactive components coelute with iodinated somatostatin added as internal marker (fig.1). One aliquot of this species was injected directly on the HPLC system and tested by RIA. The immunoreactive material was eluted as a single peak with the same retention time as the tetradecapeptide somatostatin (not shown). The 15 000 M_r material recovered after Sephadex G-50 fractionation of acid/urea extracts of hypothalamus, was pooled and the pH was adjusted to 6.0 in the presence of proteases inhibitors. If this material was immediately re-submitted to Sephadex G-50 fractionation the apparent M_r of the 15 000 M_r somatostatin-like species was found unaffected (fig.2, top). In contrast when incubated under the same conditions, for 8 h at 37°C, the

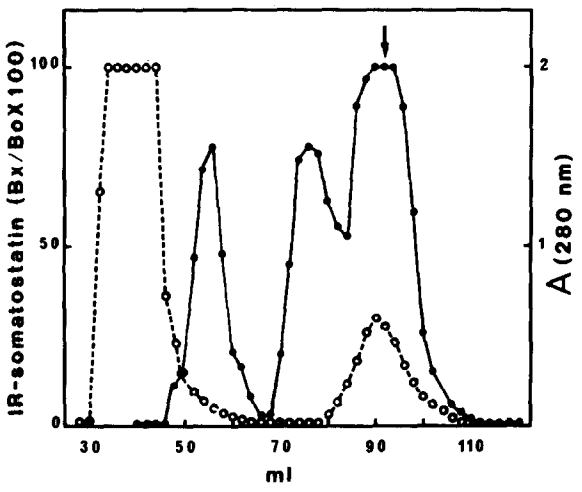

Fig.1. Fractionation of somatostatin-like forms extracted from mouse hypothalamus. The acid–urea extract was fractionated on a Sephadex G-50 column (95 × 1.2 cm) eluted with 0.1 N HCOOH containing 0.5 mM PMSF and 1 mg pepstatin/l. [^{125}I]Somatostatin was added as internal marker, its elution position is shown by the arrow. A_{280} nm (o——o) and somatostatin immunoreactivity (•——•) were measured in each fraction (1 ml). B_X/B_0 × 100 represent the percentage of antibody-bound [^{125}I]somatostatin tracer.

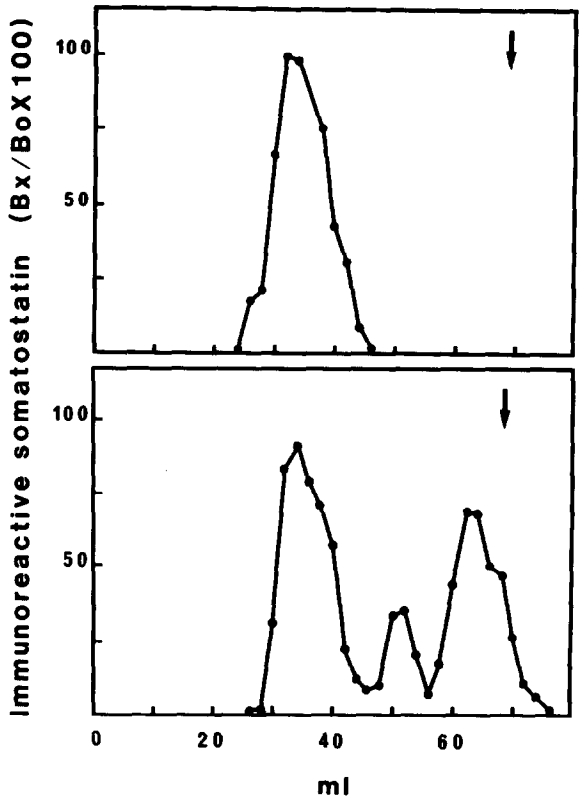

Fig.2. Processing of the 15 000 M_r prosomatostatin. The M_r 15 000 form recovered from fig.1 fractionation was resubmitted to the same chromatography as in fig.1 but on a different size column (45 × 1.2 cm) (top), and after incubation at pH 6 for 8 h at 37°C (bottom). Each fraction (1 ml) was analyzed by RIA with antiserum 3638. The arrow indicated the elution peak of [^{125}I]somatostatin used as internal marker.

pattern of somatostatin-like material recovered after gel filtration indicated that 3 species of somatostatin with M_r 15 000, 6000 and 1600 were generated (fig.2, bottom). The proteolytic activities co-eluting with the 15 000 M_r prosomatostatin apparently generated the same species as found in the crude extract. If the 15 000 M_r prosomatostatin was first purified by affinity chromatography, this species appeared stable under the conditions of the maturation experiment (not shown).

To demonstrate that at least one of the proteolytic activities coeluting with the 15 000 M_r precursors species is specific, the smaller somatostatin-like product (fig.2, bottom) was analyzed by HPLC. The material of lowest apparent M_r on Sephadex G-50 was pooled, lyophylized and redissolved in 1 M acetic

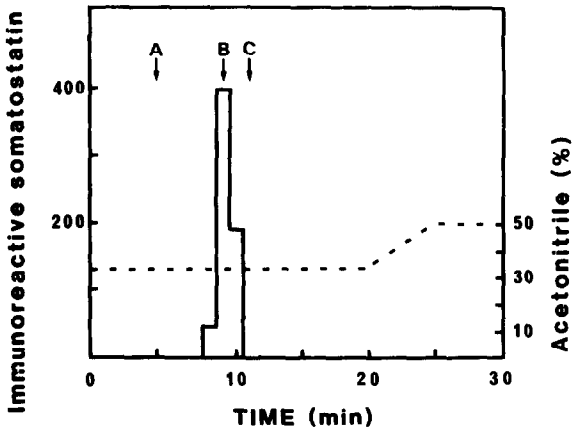

Fig.3. High-pressure liquid chromatography identification of the species generated by processing of the 15 000 M_r precursor. The fractions of somatostatin-like immunoreactivity generated in fig.2 (bottom) experiment and eluted as [^{125}I]somatostatin were pooled and analyzed by HPLC on a 10 RP 18 column (Merck) (250 × 4.6 mm) with 35% acetronile in the 50 mM acetate ammonium (pH 4) at a flow rate of 1 ml/min. Each fraction was assayed by the antisomatostatin antibodies (histogram). The arrows indicate the elution peaks of A, Tyr^{11} somatostatin B; somatostatin^{14} and C; Tyr^{N} somatostatin, respectively. Immunoreactive somatostatin was expressed in pg/fraction.

acid and injected directly on the HPLC system. The immunoreactive species were eluted as a single peak (fig.3) with a retention time indistinguishable from the one of the tetradecapeptide somatostatin I. No other material was detectable by RIA (fig.3). The presence of large quantities of trypsin inhibitors was found to be necessary to prevent the generated somatostatin from its subsequent degradation by trypsin-like enzymes (not shown).

The predicted sequences for the prosomatostatins coded by mRNA from Anglerfish pancreas indicate the presence of several Lys or Arg residues located either inside or outside the tetradecapeptide sequence; only a single dibasic stretch located at position −1, −2 (Lys −1, Arg −2), can be predicted [5,6]. Therefore, the converting enzymes must exhibit a high specificity for such a signal to release selectively the 1−14 somatostatin. If this signal appears to be the same for the prosomatostatin from mouse hypothalamus, then the above data would suggest that such a selective dibasic esteropeptidase is present in hypothalamic extracts. This enzyme would be responsible for the observed selective conversion of the 15 000 M_r prosomatostatin into the 1−14 tetradecapeptide. These data do not suggest that a second prosomatostatin is present in noticeable amounts in the hypothalamic extracts as judged by the fact that only a single 1−14 peptide could be detected after the processing of the 15 000 form. An observation consistent with those made in [8] in the pancreas. They open the way to a further identification of this processing enzyme of brain peptides precursors.

Acknowledgements

We wish to thank C. Rougeot (Unité de Radioimmunologie Analytique, Institut Pasteur) for her continuous assistance with the immunological techniques. This work was supported in part by funds from the Université Pierre et Marie Curie, the Centre National de la Recherche Scientifique (Equipe de Recherches Associée 693), the Programme Interdisciplinaire de Recherche sur les Bases Scientifiques des Médicaments, the Délégation à la Recherche Scientifique et Technique (contract 81-E-0396). The help of the Department de Biologie du Commissariat à l'Energie Atomique, Saclay for the supply of radiochemicals and a doctorate fellowship to M. L. is appreciated.

References

[1] Lauber, M., Camier, M. and Cohen, P. (1979) Proc. Natl. Acad. Sci. USA 76, 6004−6008.
[2] Spiess, J. and Vale, W. (1980) Biochemistry 19, 2861−2866.
[3] Goodman, R. H., Lund, P. K., Jacobs, J. W. and Habener, J. F. (1980) J. Biol. Chem. 255, 6549−6552.
[4] Shields, D. (1980) J. Biol. Chem. 255, 11625−11628.
[5] Goodman, R. H., Jacobs, J. W., Chin, W. W., Lund, P. K., Dee, P. C. and Habener, J. F. (1980) Proc. Natl. Acad. Sci. USA 77, 5869−5873.
[6] Hobart, P., Crawford, R., Shen, L. P., Pictet, R. and Rutter, W. J. (1980) Nature 288, 137−141.
[7] Tronquet, C., Guimbar, J.-P., Paolucci, F. (1979) in: Hormone Receptors in Digestion and Nutrition (Rosselin, G. et al. eds) pp. 89−94, Elsevier/North-Holland, Amsterdam, New York.
[8] Noe, B. D. (1981) J. Biol. Chem. 256, 9397−9400.