Presence of two molecular weight forms of somatostatin in neurons from chick embryo cerebral hemispheres in culture

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Immunoreactive somatostatin N-terminal extension of SS-14 Neuron peptide release (Chick embryo cerebral hemisphere culture)

1. INTRODUCTION

Following its isolation hypothalamic extracts [1] the tetradecapeptide somatostatin (SS-14) has been found to be widely distributed throughout the central nervous system [2], the gastrointestinal tract and the pancreas [3]. Evidence for synthesis of SS-14 by cerebral cells in culture has also been reported [4,5]. We report here that neurons from chick embryo cerebral hemispheres, cultured in serum-free medium, are able to synthetize and to release two distinct molecular forms of somatostatin: one is identical to SS-14 and the other has a similar M_r-value to, but different from SS-28, an amino-terminally extended peptide from SS-14 [6].

2. MATERIALS AND METHODS

2.1. *Cultures*

Pure cultures of neurons were obtained by the method earlier described [7]. Cerebral hemispheres of 8 day-old chick embryos were dissociated into single cells, plated on polylysine-coated dishes at a density of 7 to $8 \cdot 10^4$ cells/cm^2 and maintained in vitro for 8 days in a humidified atmosphere of 95% air–5% CO_2 at 37°C. During the first 3 days of culture the cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 20% fetal calf serum (Flow Laboratories) and antibiotics (60 U/ml of penicillin G and 25 μg/ml of streptomycin sulphate). From the 3rd to the 8th day, the serum-supplemented medium was replaced by a serum-free culture medium [8]: DMEM supplemented with human transferrin (100 mg/l), insulin (5 mg/l), putrescine (10^{-4} M), sodium selenite ($3 \cdot 10^{-8}$ M), progesterone ($2 \cdot 10^{-8}$ M) and 17β-estradiol (10^{-12} M) as described in [9]. Culture media were changed at days 3, 5 and 7. The cells and their media were collected at day 8.

2.2. *Preparation of cellular and medium extracts*

Media of 8 day-old cultures were collected in acetic acid (final concentration: 3–5 M) containing iodoacetamide (0.3 mg/ml) and phenylmethylsulfonyl fluoride (0.3 mg/ml). The cells were rinsed 3 times with 0.15 M NaCl at 37°C, collected in the same mixture as the media and homogenized with a Teflon tissue grinder. The preparations were then heated (95°C, 15 min), centrifuged (5000 rev./min, 15 min) and the supernatants were lyophilized.

2.3. *Radioimmunoassay for somatostatin*

Cell and medium extracts were analysed for somatostatin immunoreactivity by a radioimmunoassay (RIA) using anti-somatostatin antiserum (ref. no. 3638 SAB) raised in a rabbit immunized against somatostatin covalently bound to egg albumin with glutaraldehyde. [*Tyr*-^{125}I]somatostatin was used as tracer. The assay was performed at 4°C for 24 h in 0.05 M potassium phosphate buffer (pH 7.6), containing 0.15 M NaCl and 0.5% bovine albumin. Bound and free fractions were separated by propanol precipitation and the radioactivity was evaluated in a gamma-counter (70% counting efficiency). Under these conditions the detection limit was 15 pg/tube and a 50% displacement of

the tracer was obtained with 40 pg of the peptide.

Lyophilized cellular and medium extracts were redissolved in cold essay buffer. Standard somatostatins (SS-14 and SS-28) were treated in the same way as the extracts (acetic acid 3–5 M, iodoacetamide, PMSF, heat 95°C for 15 min). No modification of the immunoreactive profile was observed in these conditions.

2.4. *Chromatography*

Somatostatin-immunoreactivity was analyzed in both cellular and medium extracts after two successive gel filtrations on Sephadex G-10 and G-50 followed by ion-exchange chromatography on CM-Sephadex C-25 or DEAE-Sephadex A-25. Details of procedures are given in the legends of figs.1–3.

3. RESULTS

The cells and the media collected at the 8th day of culture (24 h after the addition of fresh medium) contained rather high amounts of immunoreactive-somatostatin (IR-somatostatin): 44 ± 2 ng/100 mm Petri dish, 48 ± 2 ng/mg protein (values are mean ± S.E. of 3 independent experiments; n = 18 determinations). No immunoreactive material was detected in serum-free culture medium. After Sephadex G-50 gel filtration, two areas of SS-immunoreactive material were obtained (fig.1) corresponding to standard SS-28 (peak I) and SS-14 (peak II). IR-somatostatin present in peak I was not retained on CM-Sephadex C-25 column, suggesting a negatively charged molecule, while the immunoreactive material found in peak II was retained and co-eluted with standard SS-14 (fig.2). IR-somatostatin of peak I was retained on DEAE-Sephadex A-25 column and eluted with 100 mM Tris buffer containing 0.2 M NaCl (88% of recovery), confirming thus the negative charge of the molecule (fig.3). The chromatographic profile indicates that the IR-somatostatin of peak I has a molecular weight of about 3000, but does not correspond to the SS-28 form, of the same molecular weight, but which is not retained on CM-Sephadex C-25 column.

The parallelism of inhibition curves for standard SS-14 or SS-28 and cellular or medium fractions (fig.4) suggests that the antigenic determinants are

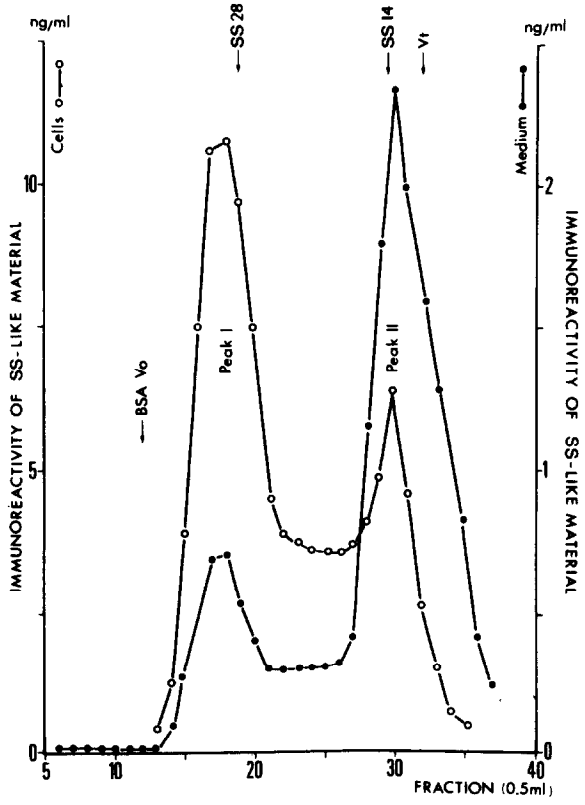

Fig.1. Sephadex G-50 gel filtration of somatostatin-immunoreactive material from cellular and medium extracts. Extracts of cells and media were desalted by Sephadex G-10 gel filtration and lyophilized. Samples for chromatography were resuspended in 1 ml of water and applied to a Biorad 0.7 × 30 cm column packed with Sephadex G-50 (fine, Pharmacia) and equilibrated with 0.01 M CH_3COONH_4 (pH 7.4), containing 0.1% BSA (Fraction V, Miles). The column was eluted with the same buffer. 0.5 ml fractions were collected at a flow rate of 20 ml/h and assayed for SS by RIA. The column was previously calibrated with BSA ($M_r \simeq 63\,000$) and standard SS-14 ($M_r \simeq 1600$) and SS-28 ($M_r \simeq 3000$). The positions of the SS peaks were determined by RIA. Void volume of column: 6 ml. (•——•) culture medium (6.9 ng IR-somatostatin applied on column); (○——○) cells (65 ng IR-somatostatin applied on column).

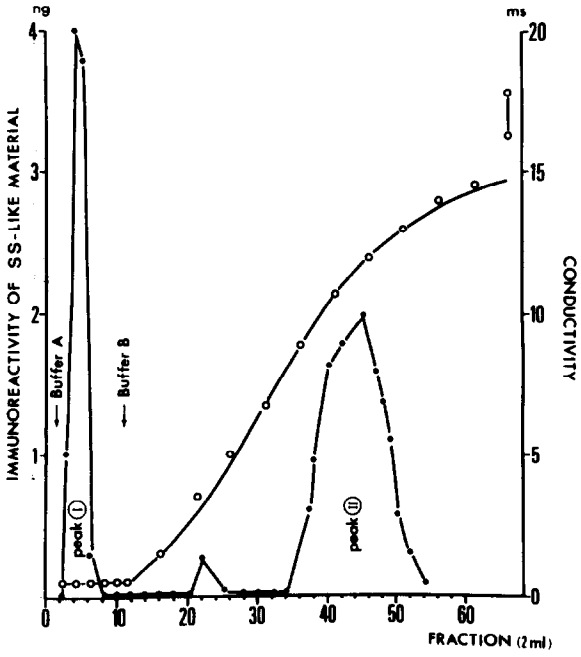

Fig.2. Chromatography of somatostatin-immunoreactive material from cellular extracts on CM-Sephadex C-25 column. Samples of peak I (corresponding to 23 ng of IR-somatostatin) and peak II (corresponding to 10 ng of IR-somatostatin) obtained by Sephadex G-50 gel filtration of cellular extracts, were lyophilized, resuspended in 6 mM CH_3COONH_4 buffer containing 0.1% BSA (pH 4.7) (buffer A) and applied to a 0.6 × 10 cm column of CM–Sephadex C-25 equilibrated with buffer A (flow rate: 20 ml/h). The column was eluted with buffer A and then subjected to from 6 mM (buffer A) to 600 mM (buffer B) $CH_3CHOONH_4$ buffer containing 0.1% BSA (pH 4.7). Two ml fractions were collected and assayed for SS by RIA. The conductivity of each fraction was determined. (•——•) SS-like immunoreactive material of the cellular extracts; (○——○) conductivity.

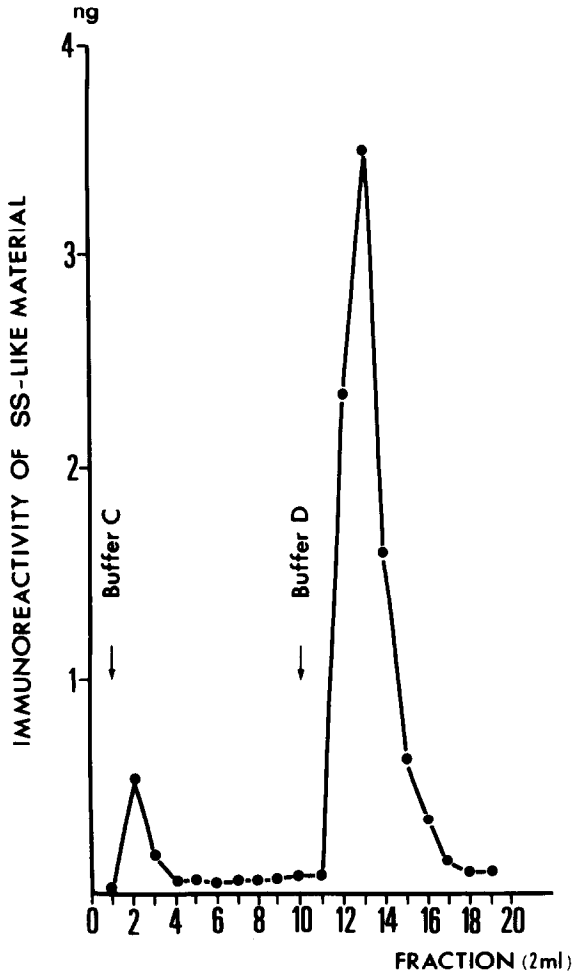

Fig.3. Chromatography on DEAE-Sephadex A-25 of the IR-somatostatin of peak I obtained by Sephadex G-50 gel filtration. Samples of peak I corresponding to 5 ng of IR-somatostatin were dissolved in 30 mM Tris–HCl (pH 8.8) (buffer C) and applied to a 0.6 × 10 cm column packed with DEAE-Sephadex A-25 equilibrated in buffer C. The column was washed (20 ml/h) with buffer C, then eluted by 100 mM Tris–HCl (pH 8.5), containing 0.2 M NaCl (buffer D). Two ml fractions were collected and assayed for SS by RIA.

similar in both molecular entities of IR-somatostatin. Moreover, treatment of the immunoreactive material of peak I with sodium dodecyl sulfate (SDS, 0.5% in 1 M acetate for 1 h at 60°C), followed by Sephadex G-50 gel filtration, yielded a unique peak of IR-somatostatin which eluted at the same position as the untreated molecule, suggesting that peak I is not formed by aggregated SS.

Peak II/Peak I (ratio of the concentrations) was 0.5 in the cells and 2.5 in the medium.

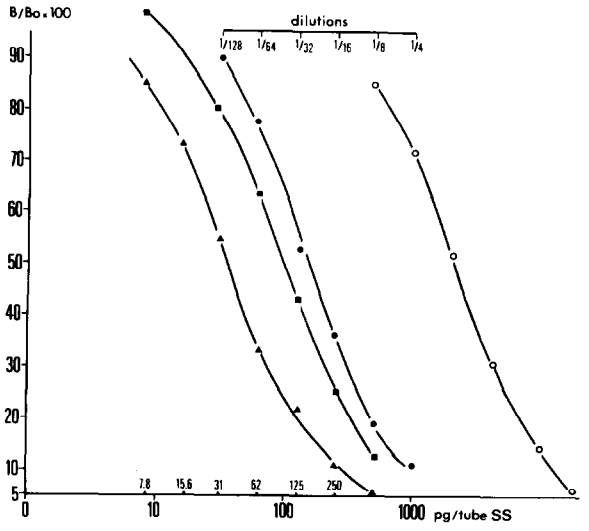

Fig.4. Inhibition curves for cellular and medium extracts in SS RIA system. Note parallelism of inhibition curves for standard somatostatins and the extracts. N.B.: standard SS-14 and SS-28 have been treated in the same way as the extracts. (▲—▲) standard SS-14; (■—■) standard SS-28; (●—●) medium extracts; (○—○) cellular extracts.

4. DISCUSSION

We have previously shown that dissociated neurons from chick embryo cerebral hemipsheres could be maintained in culture without any other cell type [7]. These neurons undergo maturation in vitro and exhibit ultrastructural and biochemical properties comparable to that of adult neurons in vivo [10,11]. The use of chemically defined culture media, without serum, offers favourable conditions to study the production of peptides by these cultures and their release into the medium: contamination by peptides present in the serum and effects of factors contained in the serum are thus avoided.

It was noteworthy that these neurons, originating from the cerebral hemispheres, were able to synthetize and to release rather high quantities of SS-like immunoreactive material. When compared to the production of IR-somatostatin by rat cerebral cells in culture [4], it appears that the amount of peptide released into the culture medium is much higher in our experimental conditions. This may be explained either by a higher rate of synthesis or release or by a lower rate of breakdown of the released SS, since the serum-free medium we use is devoid of peptidases usually present in the serum. In addition to SS, the presence of Leu-enkephalin, Met-enkephalin and substance P could be demonstrated, although at lower levels, while β-endorphin, TRH, LH-RH and ACTH could not be detected (in preparation).

Two molecular forms of IR-somatostatin could be identified: (i) the first elutes with standard SS-14 on Sephadex G-50 column and has already been described in rat cerebral [4] and hypothalamic [5] cells in culture; (ii) the second corresponds to a new molecular form of SS, of higher molecular weight ($\simeq$ 3000) and elutes in the area of SS-28, the amino terminally extended form of SS-14 recently identified in ovine hypothalamus [6,12,13] and in porcine gut [6]. However, the behaviour of this compound (negatively charged) on ion-exchange chromatography columns differs from that of SS-28, suggesting structural differences. The ratio of the quantities of the 2 molecular forms of SS in the cells and in the culture medium indicates that the high molecular weight form is preferentially retained within the cells. One might expect that this new molecular entity of SS produced by cultured neurons plays a physiological role in the processes of maturation of the neurons. Work is in progress to determine the biological activities of this new molecular entity and to define its relationship with the classical SS-14 form.

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