Multiple Forms of Somatostatin-like Activity in Rat Hypothalamus†

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ABSTRACT: Rat hypothalamic fragments were extracted by 2 N acetic acid containing protease inhibitors. The extract was subjected to acetone precipitation, defatting, and subsequent gel filtration in the presence of 6 M guanidine hydrochloride. Three species of somatostatin (SS)-like immunoreactivity were separated. The apparent molecular weights of these species as determined by gel filtration were approximately 12 000 (12K SS), 4000 (4K SS), and 2000 (2K SS). All three species were active in an SS radioimmunoassay (RIA) employing the antibody S201 directed toward the central residues in SS. In RIA's using the N-terminally directed antibody S39, 12K and 4K SS exhibited less than 5% of the activity found in RIA S201, whereas 2K SS (as is the case for synthetic SS) was equally active in both assays. The 2K species also behaved like SS in ion-exchange chromatography. The 12K, 4K, and 2K species contributed 2, 16, and 82%, respectively, to the total SS-like immunoreactivity (RIA S201) of the acetic acid extracts. Dissociation of 12K SS could not be achieved by treatment with urea, guanidine, or sodium dodecyl sulfate in the presence of reducing agent but was accomplished by urea after mild tryptic digestion, releasing a small SS-like form of SS size active in RIA S39. Growth hormone secretion from cultured rat anterior pituitary cells was inhibited by 12K SS with a relative potency of ~35% compared to immunoequivalent amounts (RIA S201) of synthetic SS. It was concluded that rat hypothalamic 12K SS represented a single chain polypeptide, which could be an SS precursor.

Somatostatin (SS)[1] has been isolated and sequenced from the ovine (Brazeau et al., 1973; Burgus et al., 1973) and porcine (Schally et al., 1976) hypothalamus and more recently from the pigeon pancreas (Spiess et al., 1979b) and the anglerfish pancreatic islet (Noe et al., 1979b). In all instances, identical primary and secondary structures have been found. Although the actions and distribution of this multifunctional polypeptide have been extensively investigated [see Vale et al. (1977) for review], our knowledge about its biosynthesis is limited.

Noe et al. (1978a,b) carried out pulse–chase experiments in the presence or absence of cycloheximide, providing evidence that SS is synthesized in anglerfish pancreatic islet by ribosomally dependent mechanisms. On the basis of the concept of Blobel & Sabatini (1970, 1971), SS may be too small for direct synthesis on the mRNA ribosome complex but could be derived from a larger precursor which would be ribosomally synthesized and then cleaved to SS in possibly several co- and posttranslational steps.

Large SS-like species have been observed in porcine (Schally et al., 1975), ovine (Vale et al., 1976; Millar, 1978; Spiess et al., 1979a), rat (Spiess & Vale, 1978; Spiess & Rivier, 1978), mouse (Lauber et al., 1979), and canine (Zyznar et al., 1979) brain, rat (Arimura et al., 1975) and canine (Zyznar et al., 1979) stomach, porcine small intestine (Pradayrol et al., 1978, 1980), pigeon (Spiess et al., 1979b; Spiess & Rivier, 1978; Spiess & Vale, 1978) and canine (Conlon et al., 1978) pancreas, anglerfish pancreatic islet (Noe et al., 1979a), and pancreatic somatostatinoma (Larsson et al., 1977).

It should be emphasized, however, that large SS-like species can only be considered as possible precursors if they withstand dissociation into smaller SS-like species by denaturing and reducing agents, since, in analogy to known prohormones (Lernmark et al., 1976; Habener, 1976), an SS precursor would be expected to be a single chain polypeptide containing the SS-like entity bound by a peptide bond. This requirement of stability under dissociating conditions has only been satisfied for a few of the reported large SS-like species.

For example, pigeon pancreatic SS-like species in the 8000–12 000-dalton range, which were stable in 8 M urea or guanidine, were almost completely dissociated into SS-like forms of SS size if reducing agent was present (Spiess & Vale, 1978; Spiess et al., 1979b). We concluded accordingly that most of the large SS-like species extracted from pigeon pancreas did not represent single chain polypeptides. These results were confirmed (Conlon et al., 1978) for a large canine pancreatic SS-like species which also dissociated only if reducing agent was present.

We present here a full documentation of our results, which establish the single chain polypeptide character of a 12 000-dalton SS-like species extracted from rat hypothalamus.

Experimental Procedures

Purification of SSLI from Rat Hypothalamus. (1) Stage I: Extraction and Subsequent Gel Filtration. Rat hypothalami (130–550) from male Sprague-Dawley rats (200–250 g) were collected immediately following decapitation and kept frozen for no more than 2 months. The frozen tissue was transferred into boiling 2 N acetic acid containing 0.1 mg/mL bacitracin (Calbiochem), 0.2 mg/mL soybean trypsin inhibitor (Sigma), and 0.01 mg/mL pepstatin A (Bachem) as protease inhibitors. A 12.5-mL amount of extraction solution was used per g of wet tissue. After 10 min, the mixture was cooled to +2 °C, homogenized with a Polytron homogenizer (Kinematica), and centrifuged initially at 4000*g* (20 min, 4 °C) and then at 28000*g* (20 min, 4 °C). This extraction procedure was repeated with ~2–4 mL of extraction solution per initial g of wet tissue. Acetone was added to the combined supernatants to a final concentration of 75% (v/v). After 10 min of

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[1] Abbreviations used: DME medium, Dulbecco modified Eagle's medium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IBMX, 3-isobutyl-1-methylxanthine; RIA, radioimmunoassay; SD, standard deviation; $NaDodSO_4$, sodium dodecyl sulfate; SEM, standard error of the mean; SS, somatostatin; SSLI, somatostatin-like immunoactivity; TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; CMC, carboxymethylcellulose.

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stirring at +4 °C, the suspension was centrifuged at +4 °C (30 min, 28000*g*). Acetone was removed from the supernatant by a stream of nitrogen gas and the supernatant was then lyophilized. The residue after lyophilization was dissolved in 1 to 2 mL of 8 M guanidine hydrochloride (Sigma) and 2 M ammonium acetate, pH 2.5, incubated in a boiling water bath (4 min) to facilitate dissociation of protein complexes by disruption of noncovalent bonds, cooled to 23 °C, and twice defatted with an equal volume of diethyl ether–hexane (2:1) each time. After defatting, samples were submitted to column (2.2 × 58 cm) chromatography employing Bio-Gel P-100. The columns were equilibrated and eluted at 23 °C by 6 M guanidine hydrochloride and 1 M ammonium acetate, pH 2.5. Calibration of these Bio-Gel P-100 columns with molecular weight markers has been described earlier (Spiess & Vale, 1978; Spiess et al., 1979b).

Elution volumes were calculated as partition coefficients K_D, referring to the internal volume of the gel [$K_D = (V_e - V_o)/(V_s - V_o)$; V_e = elution volume of the sample; V_o = void volume; V_s = elution volume of small solutes, totally included in the gel]. The fractions of the eluate were assayed by RIA's S201 and S39 prior to desalting.

In other experiments, ~50 Sprague-Dawley rats were decapitated and the hypothalami were collected, frozen on dry ice, immediately incubated in hot (100 °C) 6 M guanidine hydrochloride, 1 M ammonium acetate, and 0.2 mg/mL bacitracin, pH 2.5 (5 mL/g of wet tissue), for 10 min, homogenized with a Polytron homogenizer, cooled in ice water to ~10 °C, and centrifuged (20 min, 48000*g*). Defatting and gel filtration were performed as described above.

(*2*) *Stage II*: *Purification of Different SS-like Species Obtained by Gel Filtration of the Acetic Acid Extracts.* (*a*) *Ion-Exchange Chromatography.* Fractions of the gel filtrate of stage I with partition coefficients of $K_D = 0.79 \pm 0.1$, representing 2K SS (see Results), were desalted by gel filtration through a Bio-Gel P-2 column developed in 3 M acetic acid. Fractions containing SSLI (RIA S201) were combined and lyophilized. The lyophilizate was applied to a CM Bio-Gel A (Bio-Rad) column (0.9 × 19 cm) developed in a buffer of 0.05 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) and 1 M urea adjusted to pH 7.4 by aqueous NaOH. The column was eluted by a linear gradient between 100 mL each of equilibration buffer and 0.2 M NaCl in equilibration buffer. The eluate was assayed for SSLI by RIA S201.

This chromatographic procedure was also applied for fractions of the gel filtrate of stage I with partition coefficients of $K_D = 0.59 \pm 0.1$, representing 4K SS (see Results).

(*b*) *Gel Filtration.* Fractions of the gel filtrate of stage I with partition coefficients of $K_D = 0.21 \pm 0.1$, representing 12K SS (see Results), were desalted by dialysis in Spectrapor 3 tubing against 0.5 M acetic acid (+4 °C, 24 h). Spectrapor 3 dialysis membranes (Spectrum Medical Industries, Inc., Los Angeles, CA; nominal molecular weight cutoff 3500) were kept in boiling water (10 min) prior to dialysis. The dialyzed sample was dried by lyophilization. Aliquots of 1–3 mg of protein were incubated in 0.15 mL of 8 M urea (Schwarz/Mann, Orangeburg, NY), 0.05 M ammonium acetate, and 5% (w/v) thioglycol (pH 7.2, 24–48 h, 4 °C) and subsequently gel filtered through a Bio-Gel P-10 column (0.7 × 48 cm) developed in 3 M acetic acid (23 °C). The fractions of the eluate were lyophilized and assayed for SSLI by RIA's S201 and S39.

(*3*) *Stage III*: *Gel Filtration of 12K SS after Treatment with Sodium Dodecyl Sulfate.* Fractions obtained from the gel filtrate of stage II containing 12K SS were dried by lyophilization. An aliquot of 1.9 mg of dry weight was incubated in 0.1 mL of 5% $NaDodSO_4$, 5% (w/v) thioglycol, and 0.05 M sodium phosphate (pH 7.0, 5 min at 100 °C). The minimal weight ratio of $NaDodSO_4$ to protein was 2.6:1. The sample was then cooled to 23 °C and dialyzed in Spectrapor 3 tubing against 8 M urea, 1% (w/v) thioglycol, and 0.2 M acetic acid (pH 4.0, 23 °C, 48 h). Spectrapor 3 tubing was prepared as described above. Dialysis was continued against 6 M guanidine hydrochloride, 1 M ammonium acetate, and 1% (w/v) thioglycol (pH 2.5, 23 °C, 48 h). After this dialysis procedure, the sample was gel filtered through a Bio-Gel P-100 column (0.7 × 48 cm) equilibrated and eluted by 6 M guanidine hydrochloride, 1 M ammonium acetate, and 1% (w/v) thioglycol, pH 2.5, at 23 °C. This column was calibrated with bovine γ-globulin, cytochrome *c* (horse heart), lima bean trypsin inhibitor (type II-L, Sigma), porcine [^{125}I]glucagon (New England Nuclear), and [^{125}I-Tyr^{11}]-SS. All protein markers were pretreated with 8 M guanidine hydrochloride, 0.1 M ammonium acetate, and 5% (w/v) thioglycol, pH 7.1 (5 min, 100 °C). The exclusion limit of Bio-Gel P-100 ranged under these conditions between 19000 and 22000 daltons.

For RIA, fractions with partition coefficients of <0.54 corresponding to molecular weights of >4500 were desalted by dialysis in Spectrapor 3 as described above. Fractions corresponding to lower apparent molecular weights were desalted by gel filtration through Bio-Gel P-2 columns (0.7 × 48 cm) developed in 3 M acetic acid.

Digestion of 12K SS by TPCK–Trypsin. Fractions containing 12K SS obtained from the gel filtrations of stages II or III were combined, dried, and then dissolved in 0.1 M ammonium acetate and 10 mM $CaCl_2$, pH 7.0. TPCK–trypsin (288 units/mg, Worthington) was diluted in the same buffer and added to the fraction of 12K SS. This mixture contained 16.7 ng of TPCK–trypsin per mL and 2 ng of SS equivalents (RIA S201) per mL. The protein weight ratio of enzyme to substrate was between 1:2500 and 1:5000. The mixture was incubated at 37 °C. After defined time intervals, aliquots were withdrawn, acidified by glacial acetic acid (final concentration 20% v/v), lyophilized, and dissolved in buffer employed in the RIA's S201 and S39 (Vale et al., 1976, 1978). The buffer additionally contained 1 μg/mL soybean trypsin inhibitor. Aliquot fractions were assayed for SSLI by RIA S39. Selected lyophilized aliquots were dissolved in 160 μL of 8 M urea, 3 M acetic acid, and 1 μg/mL soybean trypsin inhibitor, pH 3.6, incubated for ~20 min at 23 °C, and then gel filtered through a Bio-Gel P-10 column (0.7 × 48 cm) developed in 3 M acetic acid and 1 μg/mL soybean trypsin inhibitor. The eluate was fractionated, lyophilized, and assayed for SSLI by RIA's S201 and S39.

Protein Determination. Protein was determined according to Lowry et al. (1951) or by amino acid analysis as described earlier (Spiess et al., 1979b).

Radioimmunoassays. SSLI was determined by radioimmunoassays of tetradecapeptide SS by using the centrally directed antiserum S201 (Vale et al., 1978) or the N-terminally directed SS antiserum S39 (Vale et al., 1976). Preparations of [^{125}I-Tyr^{1}]-SS and [^{125}I-Tyr^{11}]-SS have been described earlier (Vale et al., 1976). Peptides iodinated by the chloramine-T procedure were purified by ion-exchange chromatography on CMC followed by reverse-phase high-pressure liquid chromatography. Each sample was tested in the RIA at two or more dose levels.

SS Bioassay. The SS-like biologic activity was assayed by the inhibition of growth hormone secretion from primary rat anterior pituitary cell cultures used 4 days after plating (Vale et al., 1975).

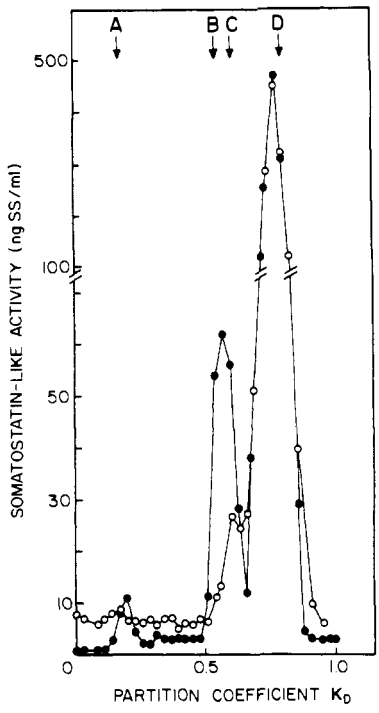

FIGURE 1: Gel filtration of the acetic acid extract of 250 rat hypothalami. The acetic acid extract (4.7 μg of SS equivalents, RIA S201) was gel filtered through a Bio-Gel P-100 column (2.2 × 58 cm) developed in 6 M guanidine hydrochloride, pH 2.5 (23 °C): RIA S39 (○); RIA S201 (●). (A) Cytochrome *c* from horse heart; (B) bovine pancreatic trypsin inhibitor; (C) porcine [^{125}I]glucagon; (D) [^{125}I-Tyr^{11}]-SS.

Results

SSLI was extracted from rat hypothalamus by hot 2 N acetic acid containing protease inhibitors. A yield of 0.71 ± 0.12 (SD) μg of SS immunoequivalents was found per g of wet tissue as determined by RIA employing the centrally directed SS antiserum S201.

Approximately 80% of the protein in this extract was precipitated by acetone, whereas most of the SSLI remained in the supernatant (Table I). This supernatant was concentrated, defatted, and subsequently gel filtered in order to separate SS-like species of different size. The overall yield of SSLI (RIA S201) after gel filtration (stage I) was ~80% (Table I).

Three distinct forms of hypothalamic SSLI, active in RIA S201, were detected by gel filtration and characterized by partition coefficients of K_D = 0.21 ± 0.01 (SD), 0.59 ± 0.03 (SD), and 0.79 ± 0.02 (SD) (Figure 1). These partition coefficients corresponded to molecular weight ranges of 11 000–12 500, 3500–4500, and 1500–2500, respectively. For convenience, we will refer to these three forms as 12K, 4K, and 2K SS. They contributed 2, 16, and 82%, respectively, to the SSLI gel filtered and assayed by RIA S201.

In SS RIA's employing the N-terminally directed antiserum S39, 12K SS showed less than 5% of the activity found in RIA S201. The S201/S39 immunoactivity ratio of fractions of 4K SS seemed to be higher than the ratio observed for 12K SS (Figure 1). However, the S39 immunoactivity of these fractions decreased after further purification by cation-exchange chromatography on CM Bio-Gel A, so that 4K SS thus purified was characterized by an S201/S39 activity ratio of 260:1.

Hypothalamic 2K SS (like synthetic SS) was equally active in both RIA's and behaved also in gel filtration (Figure 1) and subsequent cation-exchange chromatography on CM Bio-Gel A (Figure 2) like synthetic SS.

It seemed possible that relative abundance and apparent molecular weight of 12K SS obtained by gel filtration (stage I of the purification) were affected by acetone precipitation and degradation during purification and the storage period prior to extraction. Freshly collected rat hypothalami were extracted by hot 6 M guanidine and, after defatting, immediately (without acetone precipitation) gel filtered through a Bio-Gel P-100 column developed in 6 M guanidine to evaluate these possibilities. The only difference observed with this

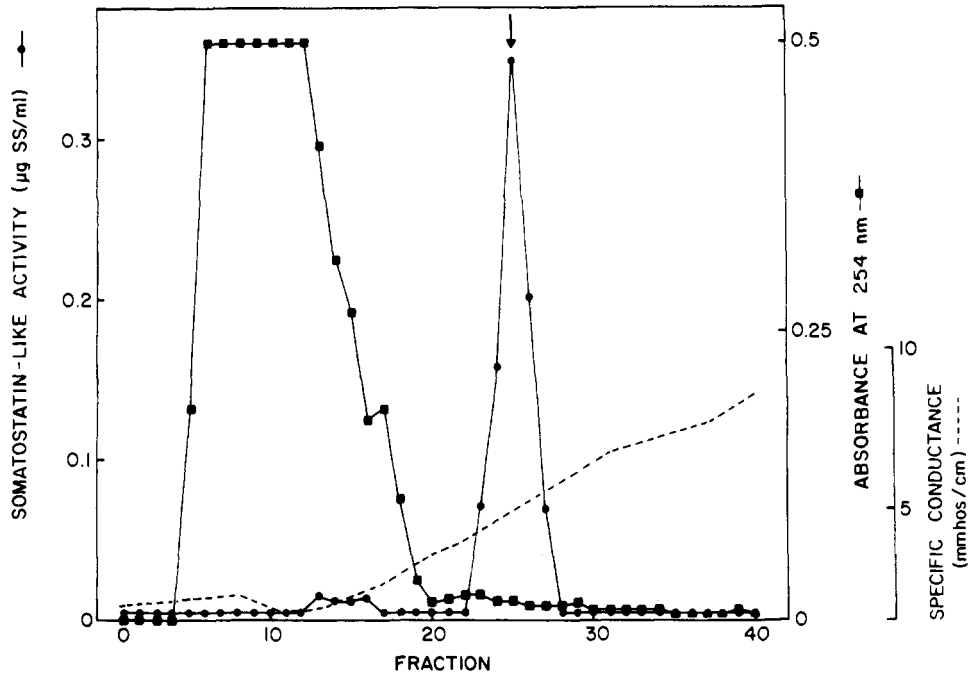

FIGURE 2: Cation-exchange chromatography of hypothalamic 2K SS on CM Bio-Gel A. An aliquot of the combined fractions from the initial gel filtration (stage I) containing 2K SS (1.7 μg of SS equivalents, RIA S201) was applied to a CM Bio-Gel A column (0.9 × 19 cm) developed in 0.05 M Hepes and 1 M urea, pH 7.4, and eluted by a linear gradient between 100 mL each of starting buffer and 0.2 M NaCl in starting buffer. The arrow marks the elution volume of SS determined in a separate run.

Table I: Initial Purification of SSLI from Rat Hypothalamus[a]

fraction	SSLI[b] (ng of SS/HT)	protein[c] (μg/HT)	sp act. (pg of SS per μg of protein)	yield (%)
crude extract	30.4	1329	22.9	100.0
supernatant of acetone pptn	24.1	222	108.6	79.3
gel filtrate	24.2	ND[d]		79.6

[a] SSLI was extracted from 130–550 hypothalami (HT) by hot 2 N acetic acid containing protease inhibitors. [b] SSLI was determined by RIA S201; each value represents the average of three to seven experiments. [c] Protein was determined according to Lowry et al. (1951). Each value represents the average of three to seven experiments. [d] ND = not determined.

procedure was an increase of the relative abundance of 12K SS in the filtrate to ~5%. No shift in apparent molecular weight was detected.

The single chain polypeptide character of 12K SS was established in experiments using thioglycol and $NaDodSO_4$ or urea as dissociating agents. Fractions of 12K SS obtained from the initial gel filtrate (stage I of the purification procedure) were incubated in 8 M urea containing 5% thioglycol and then gel filtered through a Bio-Gel P-100 column developed in dilute acetic acid. More than 80% of the SSLI applied to the column was recovered in the filtrate. In some experiments, 12K SS did not dissociate under these conditions. In other experiments, however, 12K SS partially dissociated into smaller SS-like forms varying in apparent size from experiment to experiment. The degree of dissociation never exceeded 25%.

Hypothalamic 12K SS, stable vs. urea and thioglycol, was treated with $NaDodSO_4$ under reducing conditions and subsequently, after removal of free $NaDodSO_4$, gel filtered through a Bio-Gel P-100 column developed in buffer containing guanidine and thioglycol. Approximately 65% of the SSLI submitted to this dissociation procedure was recovered in the gel filtrate. More than 70% of the activity eluted from the column was associated with 12K SS (Figure 3). The displacement curve obtained for this preparation of 12K SS in RIA S201 was parallel to the standard curve of SS. Accordingly, it was not probable that $NaDodSO_4$ had disturbed the assay, since $NaDodSO_4$ compromises such parallelism at interfering concentrations (>0.004%).

Since the possibility was considered that hypothalamic 12K SS represented an SS precursor, this polypeptide was digested with low concentrations of trypsin under conditions similar to the digestion experiments performed with proinsulin (Steiner et al., 1971) and proparathyroid hormone (Goltzmann et al., 1976). For these digestion experiments, 12K SS fractions were employed which had been previously treated by thioglycol in the presence of urea or $NaDodSO_4$ (stages II or III of the purification procedure). These fractions were incubated with low concentrations of TPCK–trypsin. As a result, SSLI detectable in RIA S39 was generated and increased during the time period recorded (Figure 4). In the absence of TPCK–trypsin, no significant change in immunoactivity (RIA S39) was observed (Figure 4). The digestive conditions were so mild that synthetic SS was not affected as determined by RIA S39 when incubated up to 48 h under identical conditions. Higher concentrations of TPCK–trypsin were required to diminish the immunoactivity of synthetic SS in RIA S39.

Partially digested 12K SS was shortly incubated in 8 M urea at room temperature and then gel filtered through a Bio-Gel P-10 column developed in dilute acetic acid containing soybean trypsin inhibitor. In the experiment presented in Figure 5, ~80% of the SSLI (RIA S39) applied to the column was

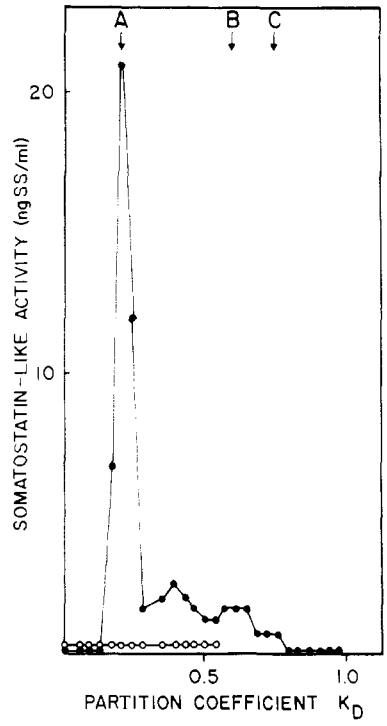

FIGURE 3: Gel filtration of hypothalamic 12K SS after incubation with $NaDodSO_4$ and thioglycol. Fractions of purification stage II (47 ng of SS equivalents in RIA S201, 1.9 mg dry weight) were incubated in 5% $NaDodSO_4$ and 5% (w/v) thioglycol (pH 7.0, 100 °C, 5 min), dialyzed, and filtered through a Bio-Gel P-100 column (0.7 × 48 cm) developed in 6 M guanidine hydrochloride and 1% (w/v) thioglycol (pH 2.5, 23 °C). The eluate was monitored by RIA S201 (●) and RIA S39 (○). (A) Cytochrome *c*; (B) [^{125}I]glucagon; (C) [^{125}I-Tyr^{11}]-SS.

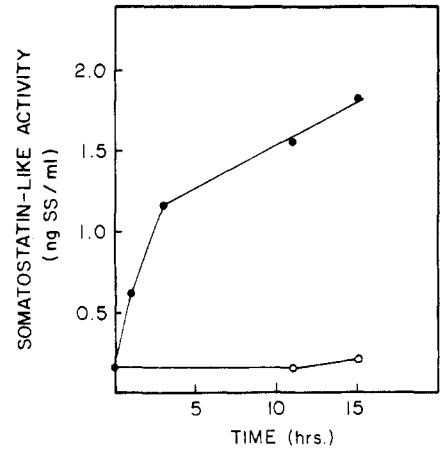

FIGURE 4: Time course of immunoactivity (RIA S39) of 12K SS after tryptic digestion. Fractions of 12K SS (stage II) were incubated at an initial concentration of 2 ng of SS equivalents (RIA S201) per mL in 0.1 M ammonium acetate and 10 mM $CaCl_2$ (pH 7.0) containing 16.7 ng/mL TPCK–trypsin. After defined time intervals, aliquots were drawn and prepared for RIA S39 (●). Control incubations were performed in the absence of TPCK–trypsin (○).

recovered in the filtrate. Most of the acitivity was associated with a small SS-like species characterized by K_D = 0.73 (Figure 5). This partition coefficient was also found for reduced SS. The ratio of activities in RIA's S39 and S201 of the small SS-like form was 2.0 ± 0.5, which is comparable to the corresponding ratio determined for reduced SS. The large SS-like form (K_D = 0.09) of this experiment (Figure 5) exhibited SSLI only in RIA S201, disappeared after more extensive digestion, and probably represented residual 12K SS. Mild trypsinization was shown to generate a small SS-like

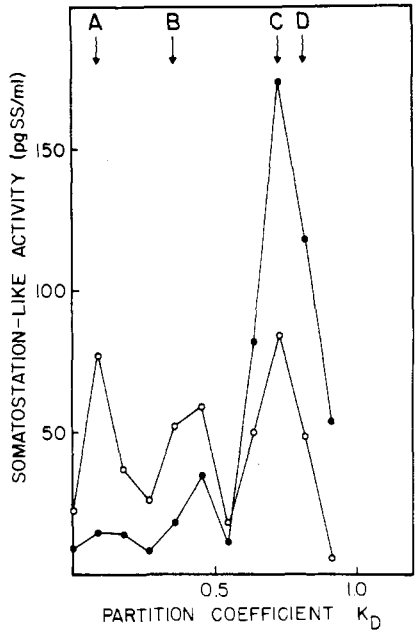

FIGURE 5: Gel filtration of 12K SS after trypsin treatment. Fractions of 12K SS (stage III) were digested by TPCK-trypsin as described in Figure 4. An aliquot after 8 h of incubation (0.64 ng of SS equivalents, RIA S39) was gel filtered through a Bio-Gel P-10 column (0.7 × 48 cm) developed in 3 M acetic acid containing 1 μg/mL soybean trypsin inhibitor. The eluate was monitored by RIA S201 (○) and RIA S39 (●). (A) Cytochrome *c*; (B) bovine pancreatic trypsin inhibitor; (C) reduced SS; (D) SS.

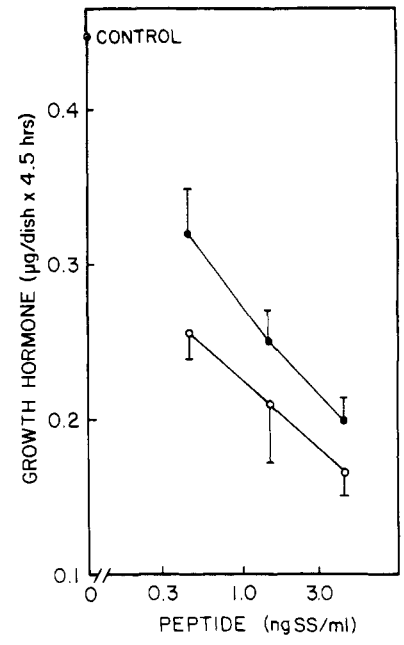

FIGURE 6: Inhibition of growth hormone secretion from cultured anterior pituitary cells (rat) by hypothalamic 12K SS of purification stage I (●) and synthetic SS (○). Primary cell cultures were plated in microwells (2×10^4 cells/microwell) and maintained for 4 days in Hepes-modified DME medium containing 10% fetal calf serum. Following a repeated change of medium, the cells were then incubated with 12K SS or SS (SS equivalents in RIA S201) in 60 μL of Hepes-modified DME medium containing 2% fetal calf serum and 0.4 mM IBMX (4.5 h, 37 °C). The medium was removed and monitored by growth hormone RIA. Each point on the dose–response curve represents three dishes. Deviations are given as the SEM.

species from 12K SS, originating from either stage II or III of the purification.

The biologic activity of hypothalamic 12K SS was determined by its ability to inhibit growth hormone release from cultured anterior pituitary cells. Fractions of the initial gel filtration were employed (stage I). At this stage of the purification, 12K SS had not been treated by reducing agents. On the basis of RIA S201, hypothalamic 12K SS was estimated to exhibit ~35% of the potency of synthetic SS (Figure 6).

Discussion

In acetic acid extracts of rat hypothalamus, three SS-like species were identified as 12K, 4K, and 2K SS. The smallest species, 2K SS, behaved like SS in gel filtration, ion-exchange chromatography, and N-terminally (S39) and centrally (S201) directed SS RIA's. These data provided evidence for rat hypothalamic 2K SS to be at least closely related to SS. Especially, the immunologic properties of 2K SS supported this conclusion, since only few SS analogues were found which exhibited the same activity with antibodies S39 and S201 (Vale et al., 1978). The primary structure of 2K SS is expected to be identical with that of SS on the basis of the finding that all small SS-like polypeptides from mammalian (Burgus et al., 1973; Schally et al., 1976), bird (Spiess et al., 1979b), or fish (Noe et al., 1979b) origin thus far isolated and sequenced were shown to have the structure of SS.

Rat hypothalamic 12K SS was tested for its stability under dissociating conditions and for immunologic and biologic activity. Whereas dissociation of hypothalamic 12K SS could not be achieved by denaturing and reducing agents alone, it could be accomplished by urea after mild tryptic digestion. These results suggested that hypothalamic 12K SS was a single chain polypeptide.

It has been established (Kemmler et al., 1971, 1973; Habener et al., 1977) that the intracellular conversion of proinsulin and proparathyroid hormone into insulin and parathyroid hormone, respectively, requires trypsin-like and carboxypeptidase B-like activities. On the basis of the experience with these prohormone hormone conversions, it seems characteristic that the trypsin-like activity can be mimicked by trypsin in very low concentrations, selectively hydrolyzing peptide bonds linking the hormone or its C-terminally extended derivative (Steiner et al., 1971; Goltzmann et al., 1976). The fact that hypothalamic 12K SS could be cleaved by TPCK-trypsin under mild conditions not affecting synthetic SS therefore supported the possibility of 12K SS being an SS precursor.

Patzelt et al. (1979) have recently reported that pulse–chase experiments with cultures of rat pancreatic islets indicated a molecular weight of 12500 for rat pancreatic prosomatostatin. In view of these results, it seemed possible that 12K SS described here represented rat hypothalamic prosomatostatin. However, only pulse–chase experiments with rat hypothalamic cells could confirm such an assumption.

The low activity of 12K SS in the N-terminally directed RIA and its tryptic cleavage to a small species exhibiting such activity suggested that 12K SS represented an N-terminally extended form of SS or of an SS-like structure. The immunologic properties of the tryptic cleavage product were compatible with reduced SS. It would not be surprising to find the reduced rather than cyclic SS in these digestion experiments since prior to trypsinization, the fractions containing 12K SS had been treated with thioglycol. However, it could not be excluded that the cleavage product was SS or an SS-like polypeptide C-terminally extended by a few (basic) residues. On the basis of this consideration, it was not possible to locate the SS-like structure more accurately on the polypeptide chain of 12K SS.

It is interesting that all large SS-like single chain polypeptides which we investigated thus far were characterized by

a high S201/S39 immunoactivity ratio. We have described this phenomenon first for a 3500–5000-dalton SS-like species purified from ovine hypothalamus (Vale et al., 1976). The single chain polypeptide character of this species has recently been established (Spiess et al., 1979a). A similar immunologic behavior was found for a 12 000-dalton SS-like single chain polypeptide extracted from rat stomach (unpublished results).

Rat hypothalamic 4K SS (this work) and a 12 000-dalton SS-like species from rat extrahypothalamic brain (Spiess & Vale, 1978), the single chain polypeptide character of which has not yet been established, also were almost exclusively active in centrally directed SS RIA's. These immunologic findings reflect structural similarities between the large SS-like species from rat and ovine hypothalamus, rat extrahypothalamic brain, and rat stomach and suggest that they all may be N-terminally extended forms of SS or an SS-like structure.

In agreement with our observations, Praydayrol et al. (1978, 1980) described an SS-like polypeptide which was isolated from porcine upper small intestine and represents an N-terminal extension of SS by 14 amino acids.

It has been shown earlier (Vale et al., 1978) that SS is one of few peptides tolerating C- and N-terminal extensions or modifications without losing biologic activity. In view of these findings, it was surprising that hypothalamic 12K SS exhibited only 35% of the biologic potency of SS in the anterior pituitary cell culture assay previously used during the isolation of ovine hypothalamic SS and for determining the biological activity of various SS analogues. This relatively low biologic potency of hypothalamic 12K SS might be explained by impaired accessibility to SS receptors of the SS-like sequence within the large polypeptide.

Attempts to purify appreciable amounts of large SS-like single chain polypeptides from bird and mammalian pancreas have not yet been successful (Dupont & Alvarado-Urbina, 1976; Spiess & Vale, 1978; Spiess & Rivier, 1978; Spiess et al., 1979b; Conlon et al., 1978). These difficulties may partially result from the proteolytic activity of exocrine pancreas present in the tissue to be extracted. This interpretation is supported by the finding that anglerfish pancreatic islets (devoid of exocrine tissue) contained and synthesized SS precursor in the 8000–15 000-dalton range, stable under denaturing and reducing conditions (Noe et al., 1978a,b; 1979a).

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References

Arimura, A., Sato, H., Dupont, A., Nishi, N., & Schally, A. V. (1975) *Science 189*, 1007–1009.

Blobel, G., & Sabatini, D. D. (1970) *J. Cell Biol. 45*, 130–145.

Blobel, G., & Sabatini, D. D. (1971) *Biomembranes 2*, 193–195.

Brazeau, P., Vale, W., Burgus, R., Butcher, M., Rivier, J., & Guillemin, R. (1973) *Science 179*, 77–79.

Burgus, R., Ling, N., Butcher, M., & Guillemin, R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 684–688.

Conlon, J. M., Zyznar, E., Vale, W., & Unger, R. H. (1978) *FEBS Lett. 94*, 327–330.

Dupont, A., & Alvarado-Urbina, G. (1976) *Life Sci. 19*, 1431–1434.

Goltzmann, D., Callahan, E. N., Tregear, G. W., & Potts, J. T., Jr. (1976) *Biochemistry 15*, 5076–5082.

Habener, J. F. (1976) *Ciba Found. Symp. 41*, 197–219.

Habener, J. F., Chang, H. T., & Potts, J. T., Jr. (1977) *Biochemistry 16*, 3910–3917.

Kemmler, W., Peterson, J. D., & Steiner, D. F. (1971) *J. Biol. Chem. 246*, 6786–6791.

Kemmler, W., Steiner, D. F., & Borg, J. (1973) *J. Biol. Chem. 248*, 4544–4551.

Larsson, L.-I., Holst, J. J., Kühl, C., Lundqvist, G., Hirsch, M. A., Ingemansson, S., Lindkaer Jensen, S., & Rehfeld, J. F. (1977) *Lancet i*, 666–668.

Lauber, M., Camier, M., & Cohen, P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6004–6008.

Lernmark, A., Chan, S. J., Choy, R., Nathans, A., Carroll, R., Tager, H. S., Rubenstein, A. H., Swift, H. H., & Steiner, D. (1976) *Ciba Found. Symp. 41*, 7–30.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Millar, R. P. (1978) *J. Endocrinol. 77*, 429–430.

Noe, B. D., Weir, G. C., & Bauer, G. E. (1978a) *Metabolism 27*, 1201–1205.

Noe, B. D., Fletcher, D. J., Bauer, G. E., Weir, G. C., & Patel, Y. (1978b) *Endocrinology (Baltimore) 102*, 1675–1685.

Noe, B. D., Fletcher, D. J., & Spiess, J. (1979a) *Diabetes 28*, 724–730.

Noe, B. D., Spiess, J., Rivier, J. E., & Vale, W. (1979b) *Endocrinology (Baltimore) 105*, 1410–1415.

Patzelt, C., Tager, H. S., Carroll, R. J., & Steiner, D. F. (1979) *Nature (London) 282*, 260–266.

Pradayrol, L., Chavialle, J. A., Carlquist, M., & Mutt, V. (1978) *Biochem. Biophys. Res. Commun. 85*, 701–708.

Pradayrol, L., Jörnvall, H., Mutt, V., & Ribet, A. (1980) *FEBS Lett. 109*, 55–58.

Schally, A. V., Dupont, A., Arimura, A., Redding, T. W., & Linthicum, G. L. (1975) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35*, 584.

Schally, A. V., Dupont, A., Arimura, A., Redding, T. W., Nishi, N., Linthicum, G. L., & Schlesinger, D. H. (1976) *Biochemistry 15*, 509–514.

Spiess, J., & Rivier, J. (1978) *Endocrinology (Baltimore) 102*, A154 (abstract).

Spiess, J., & Vale, W. (1978) *Metabolism 27*, 1175–1178.

Spiess, J., Villarreal, J., & Vale, W. (1979a) *Endocrinology (Baltimore) 104*, A294 (abstract).

Spiess, J., Rivier, J., Rodkey, J. A., Bennett, C. D., & Vale, W. (1979b) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2974–2978.

Steiner, D. F., Cho, S., Oyer, P. E., Terris, S., Peterson, J. D., & Rubenstein, A. H. (1971) *J. Biol. Chem. 246*, 1365–1374.

Vale, W., Brazeau, P., Rivier, C., Brown, M., Boss, B., Rivier, J., Burgus, R., Ling, N., & Guillemin, R. (1975) *Recent Prog. Horm. Res. 31*, 365–397.

Vale, W., Ling, N., Rivier, J., Villarreal, J., Rivier, C., Douglas, C., & Brown, M. (1976) *Metabolism 25*, 1491–1494.

Vale, W., Rivier, C., & Brown, M. (1977) *Annu. Rev. Physiol. 39*, 473–527.

Vale, W., Rivier, J., Ling, N., & Brown, M. (1978) *Metabolism 27*, 1391–1401.

Zyznar, E. S., Conlon, J. M., Schusdziarra, V., & Unger, R. H. (1979) *Endocrinology (Baltimore) 105*, 1426–1431.