Characterization of the somatostatin-like immunoreactivity extracted from an adrenal medullary tumour

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Significant amounts of somatostatin-like immunoreactivity (SLI) were detected in the extract of a human catecholamine-secreting adrenal medullary tumour. After salt fractionation and reconstitution the major portion of SLI was purified by gel filtration and two HPLC steps; in all three systems it eluted in the position of somatostatin-14. The purified somatostatin-like peptide inhibited, in a dose-related manner, growth hormone release from stimulated perfused rat anterior pituitary cells in vitro. Amino acid analysis showed the purified peptide to have an identical composition to somatostatin found in other species.

In 1973 Brazeau and colleagues isolated the tetradecapeptide somatostatin from sheep hypothalamic extracts. The primary structure has been shown to be the same in the porcine hypothalamus (Schally et al., 1976), pigeon pancreas (Spiess et al., 1979), and anglerfish islets (Noo et al., 1979b). A larger form of somatostatin, with a 14-residue N-terminal extension (somatostatin-28), has been characterized from porcine intestine (Pradayrol et al., 1980), porcine hypothalamus (Schally et al., 1980), and ovine hypothalamus (Esch et al., 1980). Immunocytochemical techniques and radioimmunoassays have been used to demonstrate the widespread distribution of somatostatin-like immunoreactivity (SLI) in the pancreas, gastrointestinal tract, and both hypothalamic and extrahypothalamic regions of the brain, in all mammalian species studied. In the peripheral autonomic nervous system SLI has also been found in some sympathetic noradrenergic neurones of guinea pigs and rats (Hokfelt et al., 1977). Furthermore, SLI has been detected by immunofluorescence techniques in human adrenal medullary tissue (Lundberg et al., 1979), while in guinea pigs, radioimmunoassay (RIA) of extracts from purified adrenal chromaffin cells has also revealed the presence of immunoreactive somatostatin (Role et al., 1981).

However, the SLI observed in adrenal medullary tissue has not been previously characterized in any species. We report here the purification from a human catecholamine-secreting adrenal medullary tumour of a peptide with the chemical and biological properties of somatostatin.

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Fig. 1. Sephadex G 50 gel filtration chromatography of the adrenal medullary tumour extract. The column (1.5 x 85 cm) was eluted with 1% formic acid, at a flow rate of 6 ml h⁻¹; 30-min fractions were collected at 4°C, monitored for u.v. absorbance at 280 nm (* * *) and assayed for SLI (□). The elution positions of the molecular-weight markers, β-LPH, ACTH, somatostatin-28, and somatostatin-14, are indicated.

Materials and Methods

Extraction and purification

A 5-g catecholamine-secreting adrenal medullary tumour was collected fresh at surgery, homogenized in 100 mM HCl, heated to 80°C, and cooled to 4°C. After addition of ammonium sulphate (10% saturation) the mixture was centrifuged for 1 h at 10 000 g. The ammonium sulphate concentration of the supernatant was increased to 80% saturation, producing a peptide-rich precipitate which after centrifugation (10 000 g, 1 h) was reconstituted in 1% formic acid and subjected to gel filtration on Sephadex G 50 (Fig. 1).

SLI was measured using a sensitive RIA following the method for unextracted samples previously described by Penman and colleagues (1979).
Further purification of the main peak of SLI from Sephadex G 50 chromatography was achieved by first subjecting the pooled fractions (132-151 ml, Fig. 1) to preparative reverse-phase HPLC (Pye Unicam, LC-XP Gradient Elution Liquid Chromatograph System) using a 10-μm Spherisorb C-18 column (1 x 10 cm), eluted at 4 ml/min, with a linear methanol gradient from 40-80% containing 1% trifluoroacetic acid (TFA). The peak fractions of SLI were partially dried down to remove methanol and finally purified by analytical HPLC (Fig. 2).

**Amino acid analysis**

Amino acid analysis was performed on two 100-μl samples from the peak fraction of SLI (11.5-11.8 min) obtained after Ultraspere propyl-CN HPLC (Fig. 2). One sample was treated with performic acid (30% H₂O₂ : 88% formic acid, 1:9) for 4 h at 2°C prior to hydrolysis to convert cysteine to cysteic acid. Both samples were
hydrolysed with 6 M HCl (200 µl) containing a crystal of phenol in evacuated Pyrex tubes at 115°C for 20 h. The hydrolysates were analysed on a Jeol JLC-6AH automatic amino acid analyser linked to a sensitive o-phthalaldehyde fluorescence detection system, according to the method of Roth (1971).

**Somatostatin bioassay**

Following previous observations (Sykes & Lowry, 1980), we elected to use a method for the determination of somatostatin bioactivity based on the ability of somatostatin to inhibit rat neurointermediate lobe extract-stimulated GH release from perfused rat anterior pituitary cells in vitro.

Ten female Wistar rats, weighing approximately 200 g, were decapitated and the pituitaries removed. Two pituitary cell columns were prepared as described by Gillies and Lowry (1978), after dividing the total harvest of anterior pituitary cells from the 10 rats into two portions. Both columns were then perfused at 37°C with Earle's balanced salt solution containing 0.25% human serum albumin, 100 U/ml aprotinin, 15 µg/ml benzylpenicillin, and 25 µg/ml streptomycin, and gassed with 95% O2 / 5% CO2.

The 10 pituitary neurointermediate lobes (NIL) which had been retained after separation from the anterior lobes were extracted in 10 ml of 10 mM HCl containing 0.9% NaCl. After centrifuging at 2000 g for 20 min, the supernatant was diluted 1 to 30 in the perfusion buffer to produce the NIL extract-containing media used for the stimulation of GH release from the anterior pituitary cell columns. Solutions of both purified and synthetic somatostatin were prepared in the NIL-containing perfusion buffer to give high, medium, and low concentrations of each, namely 50 ng/ml, 12.5 ng/ml, and 3.125 ng/ml, for synthetic somatostatin. For the purified peptide, dilutions were made from the HPLC fraction from which samples had been taken for amino acid analysis. The remaining 100 µl in the fraction was dried down under vacuum to remove the acetonitrile and TFA, reconstituted in 1050 µl perfusion buffer, and diluted further at the rate of 200 µl, 50 µl, or 12.5 µl per 2 ml of the NIL-containing perfusion buffer to obtain the three different test concentrations used.

After the two cell columns had been allowed to equilibrate for 90 min, they were stimulated every 16 min for a period of 2 min, with either perfusion buffer containing NIL extract, or perfusion buffer containing both NIL extract and somatostatin (Fig. 3). Alternate stimuli contained somatostatin, with the synthetic and purified peptides being tested on different columns. Perfusion with the high, medium, and low concentrations was randomized by following a Latin square regime; both the purified and synthetic somatostatin followed the same schedule.

Released GH in the 2-min column effluent fractions was measured using a specific rat GH RIA, with reagents kindly supplied by Dr. A. Parlow, NIAMIDD, University of Maryland School of Medicine, U.S.A. To assess the inhibitory effect of each somatostatin concentration on the perfused rat anterior pituitary cells, the percentage of NIL-stimulated GH release was determined. This was calculated after subtracting the spontaneous GH release, by dividing the GH release
Fig. 3. GH release (ng/ml) was measured by RIA in response to NIL extract alone (■), and NIL extract with somatostatin (□). Shown here is the response of the perfused rat anterior pituitary cells to (a) the purified peptide (lowest concentration), and (b) synthetic somatostatin (3.125 ng/ml). The cells were perfused at 0.5 ml/min, and stimulated for a period of 2 min every 16 min; 2-min fractions were collected.

during perfusion with NIL in the presence of somatostatin, by the mean of the two peaks of NIL-stimulated GH release that precede and follow the response to perfusion with each somatostatin concentration (Figs. 3 and 4).

Results and Discussion

The somatostain content of this human adrenal medullary tumour measured by RIA of the crude extract was 2.36 ng/mg. When the extract was subjected to gel filtration on Sephadex G 50 (Fig. 1), SLI eluted predominantly in the position of somatostatin-14. However, there was also large-molecular-weight immunoreactive material eluting prior to somatostatin-14; in particular, a small peak was observed between 73 and 81 ml, with an approximate molecular weight of 6 K.

When the SLI eluting between 132 and 151 ml from Sephadex G 50 was pooled and subjected to preparative ODS HPLC and secondly to analytical propyl-CN HPLC, the retention time of the SLI peptide corresponded to that of synthetic somatostatin-14 standard. Amino acid analysis of the peak fraction from analytical HPLC (Fig. 2) revealed an identical amino acid composition to that of hypothalamic somatostatin purified from other species (Table 1).
Fig. 4. Percent of NIL-stimulated GH release, above background, is plotted against concentration of synthetic (●) and purified (▲) somatostatin. Each somatostatin concentration was tested at least 3 times; the points represent the means ± S.E.M.).

Table 1. Amino acid composition of the peak fraction from analytical HPLC (Fig. 2).

The values are means from two determinations (± S.E.M.). Amino acid composition of somatostatin isolated from other species is shown in brackets.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Value</th>
<th>Standard Deviation</th>
<th>Species</th>
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<tr>
<td>Asp</td>
<td>1.15</td>
<td>0.12</td>
<td>(1)</td>
</tr>
<tr>
<td>Thr</td>
<td>2.20</td>
<td>0.02</td>
<td>(2)</td>
</tr>
<tr>
<td>Ser</td>
<td>1.10</td>
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<td>(1)</td>
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<tr>
<td>Glu</td>
<td>0.15</td>
<td>0.06</td>
<td>-</td>
</tr>
<tr>
<td>Pro</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gly</td>
<td>1.50</td>
<td>0.11</td>
<td>(1)</td>
</tr>
<tr>
<td>Ala</td>
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<td>0.22</td>
<td>(1)</td>
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<tr>
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<tr>
<td>Trp</td>
<td>+</td>
<td></td>
<td>(1)</td>
</tr>
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</table>

*After performic acid oxidation
Furthermore, this somatostatin-like peptide also possessed the biological properties of somatostatin-14, since both the purified and synthetic peptides produced a dose-dependent inhibition of stimulated GH release from the anterior pituitary cell columns (Fig. 4). However, the purified peptide had a lower than expected biological potency in our bioassay system. This difference can probably be accounted for by adsorption losses from such a small quantity of peptide during the drying-down procedure.

The data we have obtained from gel filtration chromatography, HPLC, amino acid analysis, and bioassay support the view that the major portion of SLI extracted from a human adrenal medullary tumour represents the authentic tetradecapeptide.

The existence of a large-molecular-weight, 6 K, form of somatostatin in the tumour extract (Fig. 1) is of interest, since SLI of this molecular size has also been reported in anglerfish pancreatic islets (Noe et al., 1979a) and mouse hypothalamic extracts (Lauber et al., 1979).

Acknowledgements

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References

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