SOMATOSTATIN ANALOGS WHICH INHIBIT GLUCAGON AND GROWTH HORMONE MORE THAN INSULIN RELEASE

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SUMMARY

Three analogs of somatostatin, [D-Cys^{14}] -, [Ala^{2}, D-Cys^{14}] - and [D-Trp^{8}, D-Cys^{14}] - somatostatin, were synthesized by the solid phase method, characterized by several means, and tested for their effects on the release of insulin, glucagon, and growth hormone. The peptides sharply suppressed the release of growth hormone *in vitro* and glucagon *in vivo*, but had less effect on insulin secretion *in vivo*. These analogs, particularly [D-Trp^{8}, D-Cys^{14}] - somatostatin, could possibly be useful for the treatment of diabetes mellitus.

Somatostatin, the growth hormone release-inhibiting hormone, has been shown to inhibit glucagon and insulin release from alpha and beta pancreatic cells by a variety of assays in several species including humans (1-9). Several authors have suggested that somatostatin, particularly in long acting form, may be potentially useful in the treatment of certain diseases including acromegaly and diabetes mellitus (8-11). Certain analogs of the somatostatin tetradecapeptide possessing some, but not all, of the actions of somatostatin should improve the therapeutic possibilities of the natural hormone; i.e. an analog exhibiting minimal suppression of insulin release but having full or enhanced glucagon and GH release-inhibiting activity might have greater potential than somatostatin for the treatment of diabetes mellitus.

Efendic et.al. (12) and Sarantakis et.al. (13) have demonstrated that some analogs of somatostatin inhibit arginine-stimulated insulin release without significantly affecting glucagon secretion. These analogs retained

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Abbreviations: GH, growth hormone; BOC, tert butyloxycarbonyl; AcOH, acetic acid.

about 10% activity for inhibiting growth hormone release compared to somatostatin. Grant et.al. (14) have since reported two additional analogs which do not significantly affect insulin or glucagon release at doses which effectively suppress GH release.

With the possibility for dissociating the effects of somatostatin established, we began a systematic search for somatostatin analogs which could suppress glucagon and GH but not insulin release, in anticipation of the potential of such agents in treating diabetes mellitus. This investigation was facilitated by the development in this laboratory of an improved *in vivo* assay for arginine-stimulated insulin and glucagon secretion in the rat (Gordin, A., Arimura, A., Meyers, C., Coy, D.H., and Schally, A.V., in preparation). We now report the synthesis and biological potencies of the first somatostatin analogs which clearly produce the desired dissociated effects on the secretion of glucagon, insulin and growth hormone.

MATERIALS AND METHODS

The peptides were synthesized stepwise by the automated solid-phase procedure (15) described previously for somatostatin (16). Reactive amino acid side chains were protected as follows: Cys, 4-methylbenzyl*; Ser and Thr, benzyl; Lys, 2-cholorcarbobenzoxy. All amino acids were coupled as their N^{α}-*tert* butyloxycarbonyl derivatives** Esterification of the first BOC amino acid to the standard polystyrene, 1% divinylbenzene resin was performed as described by Gisin (20). The tetradecapeptides were deprotected and cleaved from the resin by treatment with hydrogen fluoride containing

*Erickson et. al. (17) demonstrated the increased acid stability of the 4-methylbenzyl group over the 4-methoxybenzyl group when attached to cysteine sidechains, and they proposed its use in peptide synthesis. We prepared BOC-Cys(methylbenzyl)-OH from H-Cys(methylbenzyl)-OH (18) by the method of Ali et. al.(19) (yield, 97%; mp 70-71°; $[\alpha]_D^{25}$ -30.86, c, 0.65 in MeOH; CHN analysis calculated for $C_{16}H_{23}NO_4S$ was within 0.2% of theory). BOC-D-Cys(methylbenzyl)-OH, prepared analagously from H-D-Cys(methylbenzyl)-OH, had the same melting point and specific optical rotation ($[\alpha]_D^{25}$ +30.43, c, 0.72 in MeOH) as the L-isomer. The yields reported in Table I reflect an increase of 2-4 fold over those routinely obtained for somatostatin and analogs prepared identically, but using 4-methoxybenzyl for cysteine sulfhydryl protection. These results, along with our observation of reduced quantities of exclusion volume contaminants after routine gel filtration on Sephadex G-25, indicate the superior nature of the 4-methylbenzyl group over the 4-methoxybenzyl group for peptide synthesis.

**33% rather than 25% trifluoroacetic acid (16) was used for removal of BOC groups after each coupling cycle.

Table I. Physical Constants and Yields of Somatostatin Analogs

Analog[a]	Rf Values[b] BAW	BAWE	EPAW	iPA	$[\alpha]_D^{25}$, deg.	Yield[c] (%)
I	0.19	0.33	0.54	0.35	-22.0 (c=0.45, 0.1M AcOH)	22
II	0.11	0.35	0.56	0.40	-34.4 (c=0.52, 0.1M AcOH)	18
III	0.13	0.34	0.53	0.40	-23.4 (c=0.43, 50% AcOH)	15

[a] I, [D-Cys^{14}]-somatostatin; II, [Ala^2, D-Cys^{14}]-somatostatin; III, [D-Trp^8, D-Cys^{14}]-somatostatin.

[b] BAW, 1-butanol-acetic acid-water (4:1:5, upper phase); BAWE, 1-butanol-acetic acid-water-ethyl acetate (1:1:1:1); EPAW, ethyl acetate-pyridine-acetic acid-water (5:5:1:3); iPA, 2-propanol-1M acetic acid (2:1). The peptides were applied at 20-40ug loads to silica gel plates (Brinkman SIL-G25) and sprayed with ninhydrin and Ehrlich's reagents for visualization.

[c] Yields were calculated by comparing millimoles of peptides obtained after final purification to the total millimoles of starting BOC amino acid esterified to the resin.

10% anisole by volume for 1h at 0°. The free disulfhydryl peptides were cyclized in dilute aqueous solution at pH 7.1 by oxidation with potassium ferricyanide (16, 21). Purification of the crude lyophilized material consisted of gel filtration on Sephadex G-15 in 50% acetic acid and Sephadex G-25 in 0.2 M acetic acid, followed by partition chromatography (22) on Sephadex G-25 in the solvent system 1-butanol-acetic acid-water (4:1:5). The purified somatostatin analogs appeared homogenous by thin layer chromatography in four solvent systems (Table I), and the expected ratios were obtained from amino acid analyses of acid hydrolysates (Table II). Optical rotations and yields are reported in Table I.

GH Assays: All analogs were compared with somatostatin for their ability to inhibit the release of radioimmunoassayable growth hormone *in vitro* from enzymatically dispersed rat anterior pituitary cells prepared as described previously (23). Following four days in culture, the cells were washed and incubated for 5h at 37° in Dulbecco-modified Eagle's medium in the presence or absence of increasing concentrations of each analog or somatostatin (AY 24, 914). Growth hormone levels were determined by double antibody radioimmunoassay (24) for rat GH using the NIAMDD Rat GH RIA kit. The dose required for a 50% inhibition of GH release (ED_{50}) was calculated for each analog by the method of Rodbard (25). The potency of the analogs relative to somatostatin are reported in Table III.

Insulin and Glucagon Assays: The analogs were compared to somatostatin in their ability to inhibit the release of arginine-stimulated insulin and glucagon in anesthetized rats. Male Charles River CD rats weighing 300-350g were anesthetized with sodium amytal (10mg/100g BW) and arginine (0.5g/kg BW) was infused alone or simultaneously with somatostatin or analog for 30 minutes into the jugular vein. Blood samples were collected from the aorta 30 minutes after initiating the infusion of somatostatin or analog. Plasma levels of insulin were determined by double-antibody radioimmunoassay (26)

Table II. Amino Acid Analyses of Peptide Hydrolysates[a]

Analog	Ala	Gly	Cys	Lys	Asp	Phe	Trp	Thr	Ser	NH_3
I	0.98	1.00	1.90	2.10	1.02	3.06	0.81	1.98	0.90	0.71
II	2.00	--	1.93	1.88	1.02	2.94	0.72	2.01	0.97	1.35
III	1.00	1.02	1.98	2.10	1.01	3.12	0.83	2.00	0.91	1.07

[a]Samples were hydrolysed in 4N methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole at 110° for 18h in sealed evacuated tubes.

Table III. Biological Potency of Somatostatin Analogs Relative to Somatostatin (SS) on the Inhibition of Insulin, Glucagon and Growth Hormone Release.

	Inhibiting Activity (%)		
Peptide	Insulin	Glucagon	Growth Hormone
SS	100	100	100
I	7 (0.6-23)[a]	22 (2.6- 71)	100
II	6 (0.7-17)	81 (35-177)	230
III	10 (2.1-25)	220 (127-420)	1000

[a]95% confidence limits in parentheses.

using porcine insulin (Lilly 615-1082B1081) for labeling and a guinea pig antiserum generated against porcine insulin obtained by one of us (F.L.). Plasma glucagon levels were measured by radioimmunoassay as described by Faloona and Unger (27) using crystalline glucagon (Lilly 258-D-30-138-J) and rabbit antiserum against glucagon (Unger Lot 34, pool 2).

RESULTS AND DISCUSSION

The three somatostatin analogs (Table III) inhibited the release of glucagon and GH more than the release of insulin. [D-Cys^{14}]-somatostatin (I) was the first analog with these dissociated actions. We previously observed in two cases that multiple changes in the structure of somatostatin had an additive effect on the inhibition of GH release (28), and this prompted

the synthesis of analogs II and III. The D-Trp^8 modification was selected for its ability to increase the potency of the corresponding monosubstituted somatostatin analog in inhibiting growth hormone, insulin and glucagon secretion (29). Furthermore, this change was made in both of the previously cited examples in which the additive effect of multiple substitutions was demonstrated (28). The Ala^2 modification was based on a report which showed [Ala^2]-somatostatin to have about twice the potency of somatostatin in the growth hormone assay while showing a slight, but not statistically significant tendency toward suppressing glucagon more readily than insulin release (30).

The observed ratio of 3:1 for the selective inhibition of glucagon over insulin in I was increased to 14:1 in II and 22:1 in III. Moreover, the percent inhibition of growth hormone, which was the same for I as for somatostatin, was 2.3 and 10 times greater in II and III respectively. These results, supported by different combinations of dissociated effects observed in other analogs (12-14), indicate that somatostatin can be structurally altered to produce a significant difference between the receptor recognition capabilities of various cell types. It appears that the multiple biological effects of somatostatin can deliberately be dissociated in several potentially useful directions by suitable chemical modification.

Ideally, a somatostatin analog designed for the treatment of diabetes mellitus should; a) lower the elevated plasma glucagon levels associated with the disorder (31, 32), b) not inhibit the secretion of insulin, and c) lower the plasma growth hormone levels which increase secondarily to insulin therapy (33-36). The somatostatin analogs reported herein, particularly III, exhibit biological effects consistent with the above characteristics. Further examination of these analogs and efforts to improve and exploit their therapeutic potential are now in progress.

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