Proc. Natl. Acad. Sci. USA
Vol. 77, No. 10, pp. 6171–6174, October 1980
Medical Sciences

Synthesis and biological actions of prosomatostatin

(somatostatin/synthetic prohormone/insulin/glucagon/growth hormone)

CHESTER A. MEYERS, WILLIAM A. MURPHY, TOMMIE W. REDDING, DAVID H. COY, AND ANDREW V. SCHALLY

Endocrine and Polypeptide Laboratories, Veterans Administration Medical Center, and Department of Medicine, Tulane University School of Medicine, New Orleans, Louisiana 70146

Contributed by Andrew V. Schally, July 11, 1980

ABSTRACT **The recently isolated 28-residue sequence of prosomatostatin, a putative somatostatin precursor from pig hypothalamus and intestine, was synthesized by solid-phase methodology, characterized, and tested in rats for its effects on the release of insulin, glucagon, growth hormone, and prolactin. The synthetic product strongly suppressed plasma levels of insulin, glucagon, and growth hormone, and it appeared to be more active in the pancreas than in the pituitary. It inhibited insulin release about 5 times more effectively than somatostatin on a weight basis. The prohormone also suppressed growth hormone and prolactin levels *in vitro*. A time-course experiment for the effect of prosomatostatin on growth hormone release *in vivo* showed a significant suppression of plasma growth hormone for at least 90 min.**

The presence of larger forms of somatostatin in porcine hypothalamus (1, 2) and in rat pancreas, stomach, and duodenum (3) has been recognized, and it has been suggested that these substances may represent precursors of somatostatin (1–3). Recently, the isolation, characterization, and determination of the primary structure of putative prosomatostatin from porcine hypothalami was described (4). Hypothalamic prosomatostatin is an NH_2-terminally extended form of somatostatin that has an amino acid sequence identical to that of the porcine intestinal octacosapeptide, somatostatin-28, reported by Pradayrol *et al.* (5) (Fig. 1).

The recent successful use of the rapid solid-phase method of peptide synthesis for the preparation of comparably large biologically active peptides [e.g., β-endorphin (6, 7), vasoactive intestinal polypeptide (8), and human pancreatic polypeptide (9)], encouraged us to apply this technique to the preparation of prosomatostatin. We report here the first solid-phase synthesis and the biological actions of prosomatostatin.

MATERIALS AND METHODS

Amino acids were of the L configuration. Solid-phase synthesis was run in a Beckman model 990 peptide synthesizer. High-performance liquid chromatography (HPLC) was performed on a Waters Associates model 204 liquid chromatograph equipped with two model 6000A pumps and a model 660 gradient programmer. Amino acid analyses were run on a Beckman model 119 equipped with a system AA computing integrator. The modified single-column method used has been described (10).

Peptide Synthesis and Purification. Prosomatostatin was synthesized stepwise by using the solid-phase method described for somatostatin analogs (11, 12). The standard polystyrene/1% divinylbenzene resin was used. Amino acids were coupled as their N^{α}-*tert*-butyloxycarbonyl (Boc) derivatives, and reactive side-chains were protected as follows: Cys, 4-methylbenzyl (11); Thr and Ser, benzyl; Glu, 4-chlorobenzyl; Lys, 2-chlorocarbobenzoxy; Arg, tosyl. After attachment of the first protected amino acid to the resin (13), each amino acid was successively coupled in the presence of diisopropylcarbodiimide and, in the case of Boc-Asn, a 1 M equivalent of 1-hydroxybenzotriazole. Coupling reactions were monitored at each step by using the ninhydrin test (14) or, when Pro or Asn was at the NH_2-terminus, the chloranil test (15). Couplings that were incomplete after 2 hr were recoupled by using the appropriate preformed symmetric anhydride as described (12). Any remaining free amino groups were acetylated by using acetylimidazole (16). Removal of Boc protection at each step was accomplished by treatment for 30 min with 33% trifluoroacetic acid/CH_2Cl_2 (vol/vol) containing indole (1 mg/ml). The NH_2-terminal Boc group was removed from the completed protected peptide to avoid derivatization of Met during the HF cleavage step (17), and 0.14 mM of the product was cleaved from the resin and deprotected by using 10% anisole in HF (1 hr; 0°C). After removal of HF under N_2, the crude free disulfhydryl peptide was precipitated with ether, filtered, and dissolved in 95% AcOH (400 ml); this solution was stirred while a solution of I_2 (10-fold molar excess over peptide) in glacial AcOH (100 ml) was added dropwise over 30 min. After 4 hr, zinc powder was added, and the mixture was stirred vigorously until the orange color disappeared (W. Paleveda and S. Brady, Merck Sharp and Dohme Research Laboratories, personal communication).* The solution was immediately filtered and concentrated under reduced pressure, and the concentrate was loaded onto a column (2.5 × 95 cm) of Sephadex G-15 in 50% AcOH. The exclusion volume peak (280 nm) fractions were pooled and concentrated, and this concentrate was subjected to gel filtration on a column (2.5 × 95 cm) of Sephadex G-50 in 0.2 M AcOH. By using thin-layer cellulose chromatography as a guide, the fractions (10 ml per tube) corresponding to the main peak (280 nm) were pooled, frozen, and lyophilized. The material was then applied to a column (1.5 × 45 cm) of Whatman microcrystalline CM-cellulose and eluted by using a linear gradient formed from 300 ml each of 0.25 M NH_4Ac, pH 7 and 0.5 M NH_4Ac, pH 7.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: GH, growth hormone; HPLC, high-performance liquid chromatography; Boc, N^{α}-*tert*-butyloxycarbonyl.

* We synthesized somatostatin and several of its analogs by using this approach and compared the products with those prepared by the usual cyclization procedure (11). The compounds prepared by using this approach were identical to their conventionally prepared counterparts by HPLC, thin-layer chromatography, amino acid analysis, and several other criteria, and the yields were consistently higher.

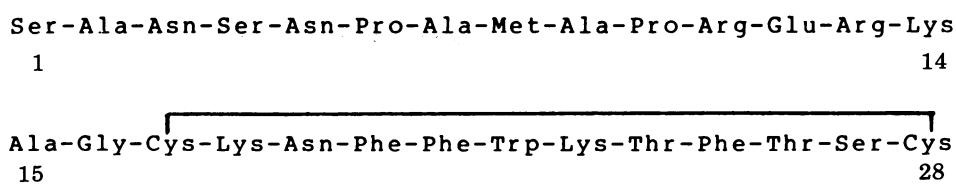

FIG. 1. Structure of prosomatostatin.

HPLC of the column fractions (5 ml per tube) was used to identify the fractions containing the major peak (280 nm), which were pooled, frozen, and lyophilized (Fig. 2). The powder was desalted on a column (2.5 × 95 cm) of Sephadex G-25 in 2 M AcOH to yield the final product.

Insulin and Glucagon Bioassays. The ability of prosomatostatin to inhibit the release of insulin and glucagon *in vivo* in male rats (CD strain, Charles River Breeding Laboratories) weighing 250–350 g was compared with that of synthetic somatostatin. The rats were kept in controlled temperature (24°C) and light (0500–1900 hr) conditions for 1 week before the assay and then fasted 27–30 hr with free access to water, and anesthetized with Nembutal (5 mg/100 g intraperitoneally). After 20 min, saline or test peptide was injected into the jugular vein; 5 min later, blood (3.5 ml) was collected from the hepatic portal vein and transferred into chilled tubes containing EDTA (2.5 mg/ml) and Trasylol (500 Kallikrein inactivator units/ml). Plasma was separated and stored at −20°C until assayed. Plasma insulin was determined by double-antibody radioimmunoassay using a kit from Cambridge Nuclear Radiopharmaceutical (Billerica, MA). Plasma glucagon was determined by the method of Faloona and Unger (18) using crystalline glucagon (Eli Lilly) and rabbit antiserum-30K against glucagon (Unger pool 4, lot 8). Porcine ^{125}I-labeled glucagon was also purchased from Cambridge. Duncan's multiple range test was used to compare the values obtained in each four-point assay (19).

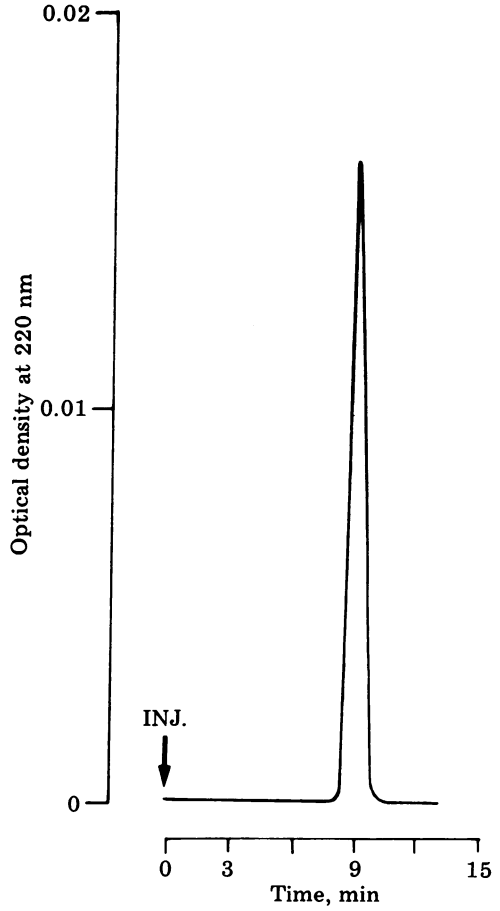

FIG. 2. Reverse-phase HPLC of synthetic prosomatostatin on LiChrosorb RP-18 (10 μm) (0.4 × 25 cm) using a linear gradient of 20–40% 2-propanol in 0.01M NH_4OAc, pH 4.0, a flow rate of 1.5 ml/min, and a load of 10 μg. Baseline corrected for changes in absorbance observed during formation of the linear gradient (blank run).

***In Vitro* Growth Hormone (GH) and Prolactin Bioassays.** The ability of prosomatostatin to inhibit the release of radioimmunoassayable GH or prolactin from enzymatically dispersed rat anterior pituitary cells was compared with that of somatostatin as described (11, 20, 21). GH and prolactin were determined by double-antibody radioimmunoassay for the respective hormones by using kits from the National Institute of Arthritis, Metabolism and Digestive Diseases.

***In Vivo* GH Bioassays: Estimation of Potency.** Male Charles River CD rats (300–350 g) having free access to food and water were anesthetized with Nembutal (5 mg/100 g intraperitoneally). After 30 min, saline or test peptide was injected subcutaneously; 15 min later, blood samples (1 ml) were drawn from the jugular vein, and the plasma was separated and assayed for GH as described above.

Time Course. Male rats were anesthetized with Nembutal and injected with saline or test substance as in the GH potency assay, and blood samples (1 ml) were collected from the jugular vein 15, 30, 45, 60, and 90 min later. Samples were treated and assayed as described above.

RESULTS

Physicochemical Data. After gel filtration chromatography of crude synthetic prosomatostatin on Sephadex G-50, the fractions (10 ml) from tubes 47–51 were pooled as described; this gave 130 mg of lyophilized powder. This was chromatographed on CM-cellulose, and the fractions (5 ml) from tubes 31–35 were pooled; on lyophilization, this gave a salt-contaminated product. Desalting of this product by gel filtration on Sephadex G-25 gave a material having a single, sharp, symmetric peak at 280 nm. The 10-ml fractions from tubes 29–37 were pooled, frozen, and lyophilized to give 21 mg of the final product (4.7% based on substitution of the first amino acid on the resin); $[\alpha]_D^{24.6} = -68.73°$ (c = 0.87, 0.1M AcOH). Amino acid

Table 1. Suppression of plasma insulin by somatostatin and prosomatostatin

Peptide	Dose, μg/100 g	Plasma insulin,* microunits per ml
None	—	83 ± 6
Somatostatin	0.4	54 ± 7
	2.0	25 ± 4
Prosomatostatin†	0.15	41 ± 9
	0.75	12 ± 1

* Mean ± SEM. n = 6 rats per group.

† Percent of somatostatin activity (95% confidence limits): 557 (268–1159).

Table 2. Suppression of plasma glucagon by somatostatin and prosomatostatin

Peptide	Dose, μg/100 g	Plasma glucagon,* pg/ml
None	—	94 ± 7
Somatostatin	0.5	79 ± 8
	2.5	63 ± 6
Prosomatostatin†	1.0	73 ± 6
	5.0	47 ± 2

* Mean ± SEM. n = 6 rats per group.
† Percent of somatostatin activity (95% confidence limits): 116 (43–411).

analysis of a sample hydrolyzed in 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole at 110°C for 48 hr in a sealed evacuated tube gave the following results: Asp, 2.91 (3); Thr, 2.12 (2); Ser 2.95 (3); Glu, 1.18 (1); Pro, 1.98 (2); Gly, 1.10 (1); Ala, 4.00 (4); Cys, 1.93 (2); Met, 0.95 (1); Phe, 3.19 (3); Lys, 2.90 (3); Trp, 0.92 (1); Arg, 1.94 (2). A single spot could be detected by using ninhydrin and Ehrlich's and Cl_2-starch spray reagents after thin-layer chromatography of prosomatostatin (20-μg loads) on microcrystalline cellulose (Whatman K2) in the following systems: 1-butanol/pyridine/AcOH/H_2O (15:10:3:12), R_F = 0.58 and EtOAc/pyridine/AcOH/H_2O (5:5:1:3), R_F = 0.59. Native prosomatostatin (4) had the identical R_F in the first system, but insufficient quantity of the native material prevented comparison in the second system. No suitable solvent system could be found for chromatography on silica gel.

Reverse-phase HPLC of synthetic prosomatostatin (Fig. 2) and of a small sample of the native material (3–5 μg) run under identical conditions (not shown) showed identical retention times.

Biological Actions. The inhibiting effects of synthetic prosomatostatin on the *in vivo* release of insulin, glucagon, and GH are compared with those of somatostatin in Tables 1–3, and the inhibiting effects of prosomatostatin on the release of GH and prolactin *in vitro* are compared with those of somatostatin in Table 4. The synthetic prohormone was strongly active in the pancreas *in vivo*, particularly in suppressing plasma insulin, where its activity was about 5 times greater than that of somatostatin on a weight basis. In the pituitary, the ability of prosomatostatin to inhibit the release of GH *in vivo* and of GH and prolactin *in vitro* was in each case about one-half that of somatostatin, also on weight basis.

The time course of the prosomatostatin suppression of plasma GH is shown in Fig. 3. At a dose of 10 μg/100 g administered subcutaneously, prosomatostatin significantly inhibited GH release for at least 90 min. The inhibitory effect of the same dose of somatostatin on GH release was not observed after 30 min (data not shown).

Table 3. Suppression of plasma GH by somatostatin and prosomatostatin

Peptide	Dose, μg/100 g	Plasma GH,* ng/ml
None	—	260 ± 81
Somatostatin	0.2	176 ± 55
	0.6	36 ± 9
Prosomatostatin†	0.4	127 ± 24
	1.2	29 ± 5

* Mean ± SEM. n = 5 rats per group
† Percent of somatostatin activity (95% confidence limits): 65 (37–114).

Table 4. Effect of somatostatin and prosomatostatin on release of GH and prolactin from rat pituitary cells in monolayer cultures

Peptide	Dose, ng/ml	Medium GH,* ng/ml	Medium prolactin,* ng/ml
None	—	3278 ± 243	213 ± 8
Somatostatin	1.0	2220 ± 159	173 ± 5
	3.0	1711 ± 50	102 ± 11
Prosomatostatin†	2.0	2215 ± 87	189 ± 8
	6.0	1825 ± 68	114 ± 13

* Mean ± SEM.
† Percent of somatostatin activity (95% confidence limits): GH, 44 (27–71) and prolactin, 40 (29–56).

DISCUSSION

This report describes the synthesis of a putative prosomatostatin recently isolated from porcine hypothalami (4). The NH_2-terminally extended form of somatostatin proved to be identical to the octacosapeptide isolated from porcine intestine by Pradayrol *et al.* (5). The preparation of synthetic prosomatostatin, in addition to providing further confirmation of the proposed primary structure for the native compound, made available sufficient material for full biological characterization and clinical testing. The biological studies using the synthetic preparation are described in this report.

The fact that synthetic and native prosomatostatin chromatographed identically in both the thin-layer and the HPLC systems (Fig. 2) suggests the correctness of the structure and also serves to demonstrate the homogeneity of the synthetic product. A further indication of the identity of prosomatostatin is that the natural and synthetic peptides were immunologically indistinguishable from each other, although both showed less affinity than somatostatin for the antiserum A101 directed toward residues 5–13 in somatostatin (4).

Prosomatostatin showed interesting biological properties. In monolayer cultures of pituitary cells, it was about half as active as somatostatin in suppressing the release of GH and prolactin on a weight basis or about equipotent with somatostatin on a molar basis. This *in vitro* finding agrees well with the activity determined for prosomatostatin on the inhibition of GH release *in vivo*. In pancreas, the putative prohormone was slightly more

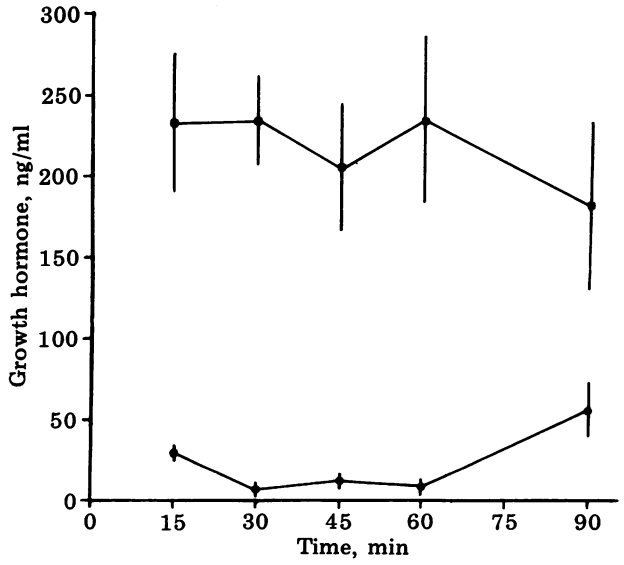

FIG. 3. Time course of plasma GH levels after injection of saline (■) or a 10 μg/100 g dose of synthetic prosomatostatin (●). Each point represents the mean ± SEM for groups of six rats.

active than somatostatin on a weight basis in suppressing plasma glucagon (about twice as active on a molar basis) and more than 5 times as active as somatostatin in inhibiting plasma insulin on a weight basis (more than 10 times as active on a molar basis). The reasons for the strong and somewhat selective actions of prosomatostatin are not known, but one possibility is that this extended form has a longer biological half-life than somatostatin. Prolonged activity (at least 90 min compared with 30 min for somatostatin) was observed in the time-course study of the effect of prosomatostatin on GH release *in vivo*.

The primary structure of putative prosomatostatin has features that are in good agreement with those of known biosynthetic peptide hormone precursors. In particular, the presence of two consecutive basic residues (-Arg-Lys-) adjacent to the somatostatin sequence (Fig. 1) is, by analogy to the established prohormones (4, 5), a likely cleavage site. Also, the two proline and two serine residues in the NH_2-terminal extension of prosomatostatin are consistent with the high frequency of occurrence of these residues in known prohormones (4). Whether the 28-residue prosomatostatin peptide represents a physiological precursor and a secretory form of somatostatin remains to be determined.

We thank Lerna D. Minor for expert technical assistance in performing the bioassays and radioimmunoassays. This work was supported by National Institutes of Health Grants AM22156 (to C.A.M.) and AM18370 (to D.H.C.) and by the Veterans Administration.

1. Schally, A. V., Dupont, A., Arimura, A., Redding, T. W. & Linthicum, G. L. (1975) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **34**, 584.
2. Schally, A. V., Dupont, A., Arimura, A., Redding, T. W., Nishi, N., Linthicum, G. L. & Schlesinger, D. H. (1976) *Biochemistry* **15**, 509–514.
3. Arimura, A., Sato, H., Dupont, A., Nishi, N. & Schally, A. V. (1975) *Science* **189**, 1007–1009.
4. Schally, A. V., Huang, W.-Y., Chang, R. C. C., Arimura, A., Redding, T. W., Millar, R. P., Hunkapiller, M. W. & Hood, L. E. (1980) *Proc. Natl. Acad. Sci. USA* **77** 4489–4493.
5. Pradayrol, L., Jornvall, H., Mutt, V. & Ribet, A. (1980) *FEBS Lett.* **109**, 55–58.
6. Coy, D. H., Gill, P., Kastin, A. J., Dupont, A., Cusan, L., Labrie, F., Britton, D. & Fertel, R. (1977) *Peptides*, Proceedings of the 5th American Peptide Symposium, eds. Goodman, M. & Meienhofer, J. (Wiley, New York), pp. 107–110.
7. Li, C. H., Yamashiro, D., Tseng, L. & Loh, H. H. (1977) *J. Med. Chem.* **20**, 325–328.
8. Coy, D. H. & Gardner, J. (1980) *Int. J. Pept. Protein Res.* **15**, 73–78.
9. Meyers, C. A. & Coy, D. H. (1980) *Int. J. Pept. Protein Res.*, in press.
10. Coy, D. H., Coy, E. J., Hirotsu, Y., Vilchez-Martinez, J. A., Schally, A. V., van Nispen, J. W. & Tesser, G. I. (1974) *Biochemistry* **13**, 3550–3553.
11. Meyers, C. A., Arimura, A., Gordin, A., Fernandez-Durango, R., Coy, D. H., Schally, A. V., Drouin, J., Ferland, L., Beaulieu, M. & Labrie, F. (1977) *Biochem. Biophys. Res. Commun.* **74**, 630–636.
12. Meyers, C. A., Coy, D. H., Huang, W.-Y., Schally, A. V. & Redding, T. W. (1978) *Biochemistry* **17**, 2326–2331.
13. Gisen, B. F. (1973) *Helv. Chim. Acta* **56**, 1476–1482.
14. Kaiser, E., Colescott, R. L., Bosinger, C. D. & Cook, P. I. (1970) *Anal. Biochem.* **34**, 595–598.
15. Christensen, T. (1979) *Peptides*, Proceedings of the 6th American Peptide Symposium, eds. Gross, E. & Meienhofer, J. (Pierce Chemical Co., Rockford, IL), pp. 385–388.
16. Markley, L. D. & Dorman, L. C. (1970) *Tet. Lett.* **21**, 1787–1789.
17. Noble, R. L., Yamashiro, D. & Li, C. H. (1976) *J. Am. Chem. Soc.* **98**, 2324–2328.
18. Faloona, G. R. & Unger, R. H. (1974) in *Methods of Hormone Radioimmunoassay*, eds. Jaffe, B. & Behrman, H. R. (Academic, New York), pp. 317–330.
19. Steel, R. G. & Torrie, J. H. (1960) *Principles and Procedures of Statistics* (McGraw-Hill, New York), pp. 107–109.
20. Gordin, A., Meyers, C. A., Arimura, A., Coy, D. H. & Schally, A. V. (1977) *Acta Endocrinol.* **86**, 833–841.
21. Vale, W., Grant, G., Amoss, M., Blackwell, R. & Guillemin, R. (1972) *Endocrinology* **91**, 562–572.