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ACCELERATED COMMUNICATIONS

Identification and Characterization of Novel Somatostatin Antagonists

ROY T. BASS, BRIAN L. BUCKWALTER, BOMI P. PATEL, MARK H. PAUSCH, LAURA A. PRICE, JOANN STRNAD,[1] and JOHN R. HADCOCK[1]

Cyanamid Agricultural Research Center, Princeton, NJ 08543-0400

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SUMMARY

The study of the five somatostatin receptor subtypes (SST_x, where *x* is the subtype number) has been hampered by the lack of high affinity antagonists. Potent and selective antagonists would increase our understanding of SST structure, function, and regulation. In this study, the identification of novel disulfide-linked cyclic octapeptide antagonists of somatostatin is described. The antagonists contain a core structure of a DL-cysteine pair at positions 2 and 7 of the peptides. Substitution of a D-cysteine at position 2 with an L-cysteine converts the full antagonist into a full agonist. All somatostatin receptor subtypes are coupled to inhibition of adenylate cyclase. The functional properties of these peptides have been determined in radioligand binding assays, in functional coupling of the SST_2 subtype to yeast pheromone response pathway, and in cAMP accumulations. One peptide antagonist [Ac-4-NO_2-Phe-c(D-Cys-Tyr-D-Trp-Lys-Thr-Cys)-D-Tyr-NH_2] displays a binding affinity to SST_2 comparable with that observed for the native hormone (K_i = 0.2 nM) and reverses somatostatin-mediated inhibition of cAMP accumulation in rat somatomammotroph GH_4C_1 cells, cells transfected with the SST_2 and SST_5 subtypes, as well as somatostatin-stimulated growth of yeast cells expressing the SST_2 subtype. This class of somatostatin antagonists, which are the first to be described, should be useful for determination of somatostatin's diverse functions *in vivo* and *in vitro*.

The cyclic tetradecapeptide somatostatin is a potent regulator of endocrine function by inhibiting the secretion of growth hormone from the pituitary, glucagon and insulin from the pancreas, and gastrin from the gut. Somatostatin also functions as a neurotransmitter and a neuromodulator in the central nervous system and peripheral tissues (1). The effects of somatostatin are transduced through binding of the hormone to high affinity, G protein-coupled somatostatin receptors (SST_x, where *x* is the subtype number) encoded in five distinct subtypes (2). These five different subtypes account for the majority of tissue-specific differences in response to somatostatin (3–9). SSTs modulate the activity of several different classes of effector molecules, including activation of potassium channels, serine/threonine and tyrosine phosphatases, Na^+/H^+ antiporters, and inhibition of calcium channels and adenylyl cyclases by transducing the signal from receptor to the effector through G proteins (10–18).

Somatostatin's central role in regulating endocrine function has made it an important research target. Many highly active analogs have been described; the comparative simplicity of many of these molecules, along with the availability of the receptor subtypes, has made somatostatin an excellent model for probing peptide-receptor interactions. Veber and his co-workers demonstrated that only four of the amino acid residues in somatostatin were required for full activity. Their general strategy has been to retain the crucial Phe[7]-D-Trp[8]-Lys[9]-Thr[10] segment (somatostatin numbering) and incorporate a variety of cyclic and bicyclic restraints to stabilize the β-type II turn about the conserved residues (19). The cyclic SST_2/SST_5-selective hexapeptide, MK-678 (Table 1), is a superagonist with 50% greater potency than somatostatin. A disulfide bond can also stabilize a β-turn. This ultimately led to the discovery and development of the potent, long-lasting somatostatin agonist SMS 201–995 (octreotide) by Bauer *et al.* (20). This octapeptide skeleton has become an excellent template for analog synthesis and a variety of potent somatostatin analogs have been prepared. Cai *et al.*, Murphy *et al.*, and Weinants *et al.* studied extensively the related disulfides and described the biological activity and conformation of numerous potent agonists (21–23). Among their most active

[1] Current affiliation: Wyeth-Ayerst Research, Princeton, NJ 08543-8000

ABBREVIATIONS: SST, somatostatin receptor; S-14, somatostatin-14; SRIF, somatostatin response inhibitory factor; FMOC, 9-fluorenylmethoxycarbonyl; DMSO, dimethylsulfoxide; TFA, trifluoroacetic acid; EDT, ethanedithiol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SC, synthetic complete; HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

TABLE 1
Structures of somatostatin peptide analogs

S-14	Ala-Gly-c[Cys-Lys-Asn-Phe^6-Phe-Trp^8-Lys-Thr-Phe-Thr-Ser-Cys]-OH
MK-678	c[N-Me-Ala-Tyr-D-Trp-Lys-Val-Thr]
SMS 201-995	H_2N-D-Phe-c[Cys-Phe-D-Trp-Lys-Thr-Cys]-Thr-ol
NC4-28-B	H_2N-D-Phe-c[Cys-Tyr-D-Trp-Lys-Ser-Cys]-Nal-NH_2
RC-160	H_2N-D-Phe-c[Cys-Tyr-D-Trp-Lys-Val-Cys]-Thr-NH_2
BIM-23066	H_2N-D-Phe-p-NO_2-Phe-Tyr-D-Trp-Lys-Val-Phe-Thr-NH_2
1	H_2N-4-NO_2-Phe-c[Cys-Tyr-D-Trp-Lys-Val-Cys]-Tyr-NH_2
2	H_2N-4-NO_2-Phe-c[D-Cys-Tyr-D-Trp-Lys-Val-Cys]-Tyr-NH_2
3	AcNH-4-NO_2-Phe-c[Cys-Tyr-D-Trp-Lys-Thr-Cys]-Tyr-NH_2
4	AcNH-4-NO_2-Phe-c[D-Cys-Tyr-D-Trp-Lys-Thr-Cys]-Tyr-NH_2
5	H_2N-4-NO_2-D-Phe-c[D-Cys-Tyr-D-Trp-Lys-Val-Cys]-Tyr-NH_2

agonists were RC-160 (21) and NC4-28-B (23) (Table 1), which are closely related to octreotide. More recently, Raynor *et al.* have shown that linear peptides (e.g., BIM-23066) also can bind to the somatostatin receptor, and these compounds exhibit subtype selectivity (24). Throughout the development of these highly potent somatostatin ligands, however, none has yet been described that is an effective antagonist of somatostatin. Potent antagonists would be a useful pharmacological tool for studying somatostatin actions and potentially enhancing the secretion of growth hormone, insulin, and other hormones.

Experimental Procedures

Materials. Protected amino acids and 1-hydroxy-benzotriazole were purchased from SNPE, Inc. (Princeton, NJ); FMOC-*p*-nitrophenylalanine and FMOC-D-tyrosine were purchased from Bachem Bioscience (King of Prussia, PA); FMOC-D-cysteine was purchased from Advanced Chemtech (Louisville, KY); dimethylformamide was purchased from Baxter (Edison, NJ); piperidine, TFA, EDT, and diisopropylyethylamine were purchased from Aldrich Chemical (Milwaukee WI); BOP was purchased from Richealieu Biotechnologies (Montreal, Quebec, Canada); anhydrous ether and DMSO were purchased from J. T. Baker (Phillipsburg, NJ); leupeptin was purchased from Bachem (Philadelphia, PA); aprotinin, bacitracin, and benzamidine were purchased from Sigma (St. Louis, MO) and ^{125}I-Tyr^{11}S-14 was purchased from Amersham (Arlington Heights, IL).

Peptide synthesis. The automated, continuous-flow, solid-phase synthesis of peptides was carried out on FMOC-PAL-PEG resin (Millipore, Burlington, MA) in a Millipore 9050 Peptide Synthesizer by standard protocols. Free peptides (0.3 mmol) were cleaved from the resin by stirring in 30 ml of 5% EDT in TFA for 4 hr at 25°. The resin was removed by filtration, the TFA evaporated and the crude peptide was precipitated with anhydrous ether.

The cyclic disulfide was formed by stirring the crude peptide in 250 ml of DMSO (25) (20–50%) in water for 24–48 hr at 25° until the solution was negative to Ellman's reagent (26). DMSO and water were evaporated and the crude cyclic peptides were purified by high performance liquid chromatography on a C18 column (300 Å, 12 mm, 21.4- × 250-mm column (Rainin Instruments, Woburn, MA) with a linear gradient of acetonitrile in 0.1% TFA in water. The fraction containing the peptide was evaporated and the residue was lyophilized. Homogeneity was confirmed by reversed-phase analytical high performance liquid chromatography (>98%) and electrospray mass spectrometry. Yields ranged from 9% to 27%.

Cloning and expression of somatostatin receptor subtypes. The cloning and expression of rat SST_1, SST_2, and SST_5 have been described previously (15, 27, 28). Rat SST_3 cDNA was cloned using a polymerase chain reaction-based strategy.[2] SST_1, SST_2, and SST_3 were expressed in Chinese hamster ovary cells. SST_5 was expressed in HEK 293 cells.

[2] R. T. Bass, B. L. Buckwalter, B. P. Patel, M. H. Pausch, L. A. Price, J. Strnad, and J. R. Hadcock, manuscript in preparation.

Membrane preparation. Crude plasma membranes were prepared according to Hadcock *et al.* (29). Cells were detached from plates with phosphate-buffered saline, pH 7.4, and 2 mM EDTA and centrifuged at 1500 × *g* for 10 min at 4°. Cells were then resuspended in 10 ml of homogenization buffer (1 mM sodium bicarbonate, pH 7.4, 1 mM EDTA, 1 mM EGTA, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 100 μg/ml bacitracin, 100 μg/ml benzamidine) for 10 min on ice. The cells were lysed in a glass/glass dounce homogenizer (20 strokes). The lysates were centrifuged at 1500 × *g* for 10 min at 4°. The supernatant was centrifuged at 40,000 × *g* for 20 min at 4°. This step was repeated twice. The pellet was then resuspended at 1–5 mg/ml in binding buffer (50 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 0.25% bovine serum albumin) and stored at -80°.

Radioligand binding. All radioligand binding assays were performed in 96-well microtiter plates using binding buffer that contained protease inhibitors (5 μg/ml leupeptin, 5 μg/ml aprotinin, 100 μg/ml bacitracin, and 100 μg/ml benzamidine). ^{125}I-Tyr^{11}S-14 (150,000 cpm at 2,000 Ci/mmole, 250 pM final concentration). The final volume was 200 μl/well. All incubations were carried out at 25° for 2 hr. Free radioligand was separated from bound ligand by rapid filtration through a glass fiber filter (IH-201-HA) using an Inotech cell harvester. The filter was then washed several times with cold (4°) binding buffer without bovine serum albumin and protease inhibitors before counting in an LKB γ master counter (Wallac, Gaithersburg, MD) (78% efficiency).

cAMP accumulations. cAMP accumulation was measured in intact GH_4C_1 cells and cells that express SST_2 and SST_5 subtypes as described previously (27). Inhibition of cAMP accumulation was measured in the presence of 10 μM forskolin. Cells were detached from plates and suspended in Krebs-Ringer phosphate buffer containing 2 mM $CaCl_2$ and 100 μM isobutylmethylxanthine. Reactions (50,000 cells per assay tube) were allowed to proceed for 15 min at 37° and were terminated by the addition of 1 N HCl. The samples were then heated for 3 min at 100° and neutralized with 1 N NaOH. Amounts of cAMP in each sample were determined as described by Hadcock *et al.* (30).

Yeast expression. Assay of stimulation and inhibition of growth of yeast in response to somatostatin receptor agonists and antagonists was performed as described (31) except that 0.2% sucrose was added to improve growth conditions. LY268 cells were plated in agar medium in the absence (agonist plate) or presence (antagonist plate) of 100 nM S-14. Somatostatin analogs (in 5 μl of DMSO) were applied to the plates directly or on sterile filter disks. The plates were then incubated at 30° for 48 hr.

Data analysis. All data were analyzed using GraphPAD Prism (San Diego, CA) and are presented as the mean ± standard deviation unless otherwise noted. K_i values were determined using the equation of Cheng and Prusoff (32).

Results and Discussion

Raynor *et al.* reported recently a series of linear peptides that exhibited subtype selectivity (24). One of these analogs, BIM-23066 (Table 1), contained a 4-NO_2-Phe residue. BIM-

23066 has an affinity for the SST_2 that is comparable with related peptides in described in Table 1 but had a very weak effect on growth hormone levels. We believe this suggests an analog with potential antagonist properties. In earlier work in our laboratories with related D-Cys^1,Cys^8 cyclic octapeptides, compounds that ultimately proved to be agonists, we noted that substitution of the aromatic side-chain residues of position 2 or 3 of these related analogs frequently led to substantial increases in affinity for the SST_2.

We decided to investigate the effect of substitution of the corresponding aromatic side chains in the related Cys^2, Cys^7 octapeptide series (**1-5**). Bauer *et al.*, Cai *et al.*, Murphy *et al.*, and Weinants *et al.* have shown that this backbone is an effective ligand for the SST_2. In addition to a clearly demonstrated utility in the somatostatin series, we were intrigued by the apparent utility of this scaffold for preparing agonists and antagonists of a variety of other G protein-coupled peptide receptors. Hruby *et al.* developed oxytocin antagonists that use a cyclic disulfide scaffold (33). Pelton *et al.* (34) and Walker *et al.* (35) have independently demonstrated that molecular modification can convert a potent cyclic disulfide somatostatin agonist with weak binding to opioid receptors into potent μ-selective opioid antagonists with little residual somatostatin activity. More recently, Orbuch *et al.* followed a similar strategy to convert a somatostatin agonist into a neuromedin B antagonist (36). The last two receptors were particularly interesting because their endogenous ligands are linear peptides, not cyclic disulfides. Thus, there seems to be sufficient structural homology among these seven membrane-spanning G-protein-linked peptide receptors to accommodate the 20-membered cyclic peptides at the binding site; receptor selective agonist/antagonist properties can be conferred by optimizing the side-chain structure and the stereochemistry of the amino acids. The role of the side chains in relation to the peptide backbone has been explored and emphasized by several groups (37, 38).

We report herein the preparation of two pairs of cyclic disulfide somatostatin analogs, **1-2** and **3-4**, which differ primarily at position 6 (octapeptide numbering). The former pair have a valine residue at position 6, whereas the latter have a threonine residue and the amino terminus is acetylated. Both valine and threonine are known to produce somatostatin agonists; valine generally exhibits higher affinity binding (39). *N*-acetylation did not qualitatively alter their properties.

Three different assays were used to characterize the pharmacological properties of these peptide analogs *in vitro*: competition binding assays with individual somatostatin receptor subtypes (Table 2), cAMP accumulation assays in rat GH_4C_1 somatomammotroph cells and cells that express the SST_2 and SST_5 subtypes (Table 3), and a novel yeast-based expression system for SST_2 subtype (31).

Competitive binding with ^{125}I-Tyr^{11}S-14 to cloned SST subtypes. Both pairs (**1-2** and **3-4**) of disulfide-linked somatostatin ligands (Table 1) bound SST_2 and SST_5 subtypes. Three of the peptides, **1**, **3**, and **4** (Table 2) all bind to the SST_2 with affinity comparable with that of S-14. Only **2**, which contained the D-Cys^2, L-Cys^7, and Val^6 exhibited weaker binding affinity than did S-14. Likewise **2**, **3**, and **4** all bind to the SST_5 with affinity better than that of S-14. The K_i values for all the compounds at both subtypes ranged from 0.1 to 18 nM. In the Val^6 series, the L-Cys,L-Cys analog **1** had much higher affinity for both subtypes than the D-Cys,L-Cys peptide **2**. There was essentially no selectivity for either subtype. In the Thr^6 series, however, the stereochemistry of Cys^2 had little effect on receptor affinity for both subtypes. For **3** and **4**, there was a distinct preference for the SST_2. Inversion of the configuration at the first residue (**2** versus **5**) caused a marked increase in the affinity for the SST_5 compared with the SST_2. In contrast, the binding affinities of the analogs was poor against SST_1 and SST_3 subtypes. In general, the K_i values were in the high nanomolar to low micromolar range for SST_1 and SST_3.

Somatostatin receptor subtypes have been subdivided into

TABLE 2

Binding affinities to SST subtypes for peptide analogs in the current study

Membranes from CHO or HEK 293 cells expressing the SST_2 (CHO), SST_3 (CHO), and SST_5 (HEK 293) subtypes were prepared. The ability of increasing concentrations of each compound (10^{-12} M to 10^{-6} M) to displace ^{125}I-Tyr^{11}-S-14 (50 fmol, 250 pM) was examined in membranes prepared from the cell expressing each subtype. For each value, 3 μg of protein/tube was used for SST_2 and SST_3, and 10 μg of protein/tube for SST_1 and SST_5. Displayed are the means of two or three determinations, each performed in duplicate. The standard deviations for each determination are <10% of the mean.

Compound	Radioligand binding			
	SST_1	SST_2	SST_3	SST_5
	K_i (nM)			
S-14	0.2	0.2	0.1	5
1	$>10^3$	0.2	300	0.1
2	$>10^3$	12.9	$>10^3$	18.1
3	$>10^3$	0.1	$>10^3$	2.2
4	$>10^3$	0.3	10^2	2.5
5	n.d.	17.3	n.d.	0.42

n.d., not determined.

TABLE 3

Inhibition of cAMP accumulation in intact GH_4C_1, CHO, and HEK cells and yeast data for peptide analogs

The values for inhibition of cAMP accumulation for the peptides at 1 μM concentration in GH_4C_1 cells, CHO cells transfected with the SST_2 receptor, and HEK cells transfected with SST_5 receptors are presented relative to that observed for 1 μM S-14, where the activity of 1 μM S-14 is 1. Relative activity = $[cAMP]_{forskolin} - [cAMP]_{peptide}/[cAMP]_{forskolin} - [cAMP]_{S\text{-}14}$. Yeast activity was scored on a qualitative 1–5 scale based on the radius of the zone of growth (agonist screen) or lack of growth (antagonist screen). Positive numbers were active in the agonist screen and negative numbers were active in the antagonist screen. All determinations are the means of at least three independent experiments. The standard deviations for each determination are <10% of the mean.

Compound	cAMP accumulation			Yeast
	GH_4C_1	SST_2 (CHO)	SST_5 (HEK)	
S-14	1.00	1.00	1.00	+5
1	0.73	0.81	0.94	+3
2	0.11	0.12	0.29	−5
3	0.95	1.02	1.00	+4
4	0.03	0.08	0.20	−5
5	0.42	n.d.	n.d.	−2
2 + SRIF[a]	n.d.	0.16	0.51	
4 + SRIF[a]	n.d.	0.10	0.36	
Basal[b]	6 ± 3	10	6	
Forskolin[c]	110 ± 15	147	185	
SRIF + forskolin[d]	44 ± 9	88	115	

n.d., not determined.

[a] 1 μM peptide + 10 nM S-14.

[b] Control cAMP levels (pmol/10^6 cells ± standard deviation) for three experiments.

[c] Forskolin (10 μM) stimulated cAMP levels (pmol/10^6 cells ± standard deviation).

[d] Reversal of forskolin (10 μM) stimulated cAMP levels by 1 μM S-14 (GH_4C_1) or 10 nM S-14 (SST_2 or SST_5) (pmol/10^6 cells ± standard deviation).

two groups: SRIF subgroups 1 (SST_2, SST_3, SST_5) and 2 (SST_1, SST_4). The work of Raynor *et al.* (24) has shown that the SST_3 subtype has an intermediate affinity for short peptide analogs compared with SST_1 (poor affinity) and SST_2 (high affinity). Our findings are in agreement with these observations: we have found that these peptides exhibit high affinity for the SST_2 and SST_5 and much lower affinity for the SST_1 and SST_3 subtypes.

cAMP data. To determine the functional properties of these somatostatin ligands, cAMP accumulations were performed in rat GH_4C_1 somatomammotroph cells (Table 3; Fig. 1). Based upon purification of somatostatin receptors from rat GH_4C_1 cells, SST_2 is the predominant subtype expressed by these cells (40). The peptides that contained a L-Cys^2,L-Cys^7 pair, **1** and **3**, inhibited forskolin-stimulated cAMP accumulation (Table 3) with efficacy similar to that observed for S-14, which suggests that both **1** and **3** were full agonists. To further characterize the functional properties of **1** and **3**, dose-dependent inhibition of cAMP accumulation was performed in GH_4C_1 cells and both analogs inhibited forskolin-stimulated cAMP accumulation in a dose-dependent manner. Maximal activity was comparable with that of somatostatin (Fig. 1, B and D).

In contrast, the compounds that contained a D-Cys^2,L-Cys^7 pair, **2** and **4**, caused little inhibition of cAMP accumulation (11% and 3% of S-14 activity, respectively) despite exhibiting high affinity binding to the SST_2. Dose titrations (Fig. 1, A and C; *dark bars*) indicated that **2** and **4** exhibit little, if any, agonist activity. However, when the dose titrations were carried out in the presence of somatostatin (Fig. 1, B and D; *light bars*) both readily reversed S-14 mediated inhibition of cAMP accumulation in a dose-dependent manner, which suggests that these two analogs are, in fact, somatostatin antagonists. Similar results were obtained in GH_4C_1 cells that express the rat A_{2a} adenosine receptor and with use of adenosine agonists rather than forskolin to stimulate cAMP accumulation (data not shown).

The effect of the two antagonists on somatostatin-mediated inhibition of cAMP accumulation was examined in S-14 dose-response curves. In the absence of antagonist, ED_{50} values

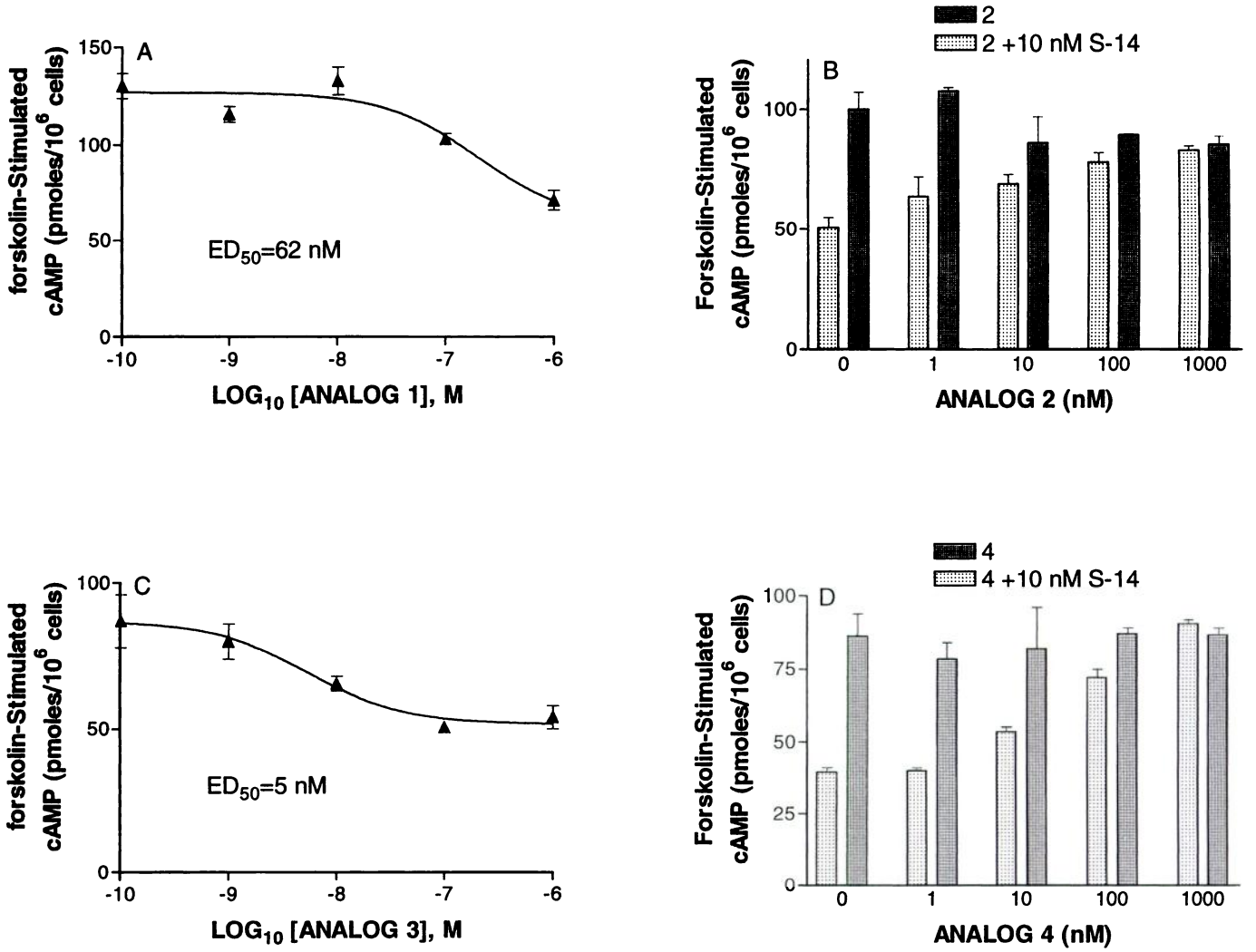

Fig. 1. Comparison of the functional properties of somatostatin analogs in GH_4C_1 somatomammotroph cells. The ability of somatostatin analogs **1-4** to inhibit forskolin-stimulated cAMP accumulation in intact cells was examined. Rat GH_4C_1 somatomammotroph cells were challenged with 5 μM forskolin and increasing doses of analogs **1** (A), **2** (B), **3** (C), and **4** (D). Analogs **2** and **4** were also tested in the presence of 10 nM S-14 (B and D). cAMP accumulations were performed as described in Experimental Procedures. Displayed is a representative experiment performed either two times (analogs **1** and **3**) or three times (analogs **2** and **4**) with equivalent results.

(half-maximal inhibition of the S-14 response) of somatostatin-mediated inhibition of cAMP accumulation was calculated to be 10 nM (Fig. 2, A and B). The presence of **2** (1 μM) shifted the S-14 dose response curve to the right by 40-fold, to 0.4 μM, whereas the presence of **4** (1 μM) shifted the S-14 dose response curve to the right by 500-fold, to 6 μM. From these curves, ED_{50} values were calculated to be 90 nM for **2** and 15 nM for **4**. Neither analog decreased the maximal inhibition of cAMP accumulation mediated by S-14, which suggests that both **2** and **4** are acting as competitive antagonists.

Analogs **1-4** were also tested for their ability to inhibit forskolin-induced elevation of cAMP in CHO cells transfected with SST_2 and in HEK 293 cells transfected with SST_5 (Table 3). In general, the results parallel those observed in the GH_4C_1 cells. Both **2** and **4** reversed S-14 antagonism of forskolin-induced elevation of cAMP. The reversal was more

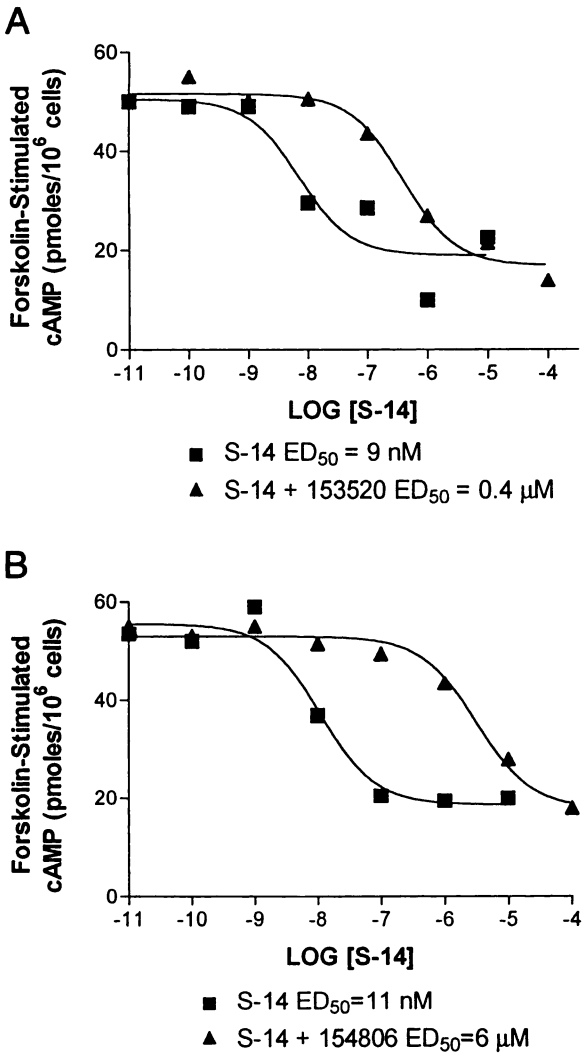

Fig. 2. Somatostatin analogs **2** and **4** reverse the dose-dependent inhibition of cAMP accumulation by S-14 in rat GH_4C_1 somatomammotroph cells. The ability of analogs **2** and **4** to antagonize S-14 mediated inhibition of cAMP accumulation was examined in rat GH_4C_1 somatomammotroph cells. Cells were challenged with 5 μM forskolin and increasing concentrations of S-14 (10^{-10} to 10^{-4} M) in the absence or presence of 1 μM analog **2** (A) or analog **4** (B). cAMP accumulations were performed as described in Experimental Procedures. Displayed is a representative experiment performed three times with equivalent results.

complete with the SST_2 subtype. These peptides may be showing some partial agonist activity at the SST_5.

The key difference in both pairs of compounds is the stereochemistry of Cys^2: D-Cys^2 analogs are antagonists at the SST_2, whereas L-Cys^2 analogs are agonists. A second important element required for antagonist activity is the stereochemistry, but not the substitution, on Phe^1. Replacement of 4-NO_2-Phe^1 with 4-NO_2-D-Phe^1 produced peptide **5**, which had properties similar to **2** in the cAMP accumulation assay but was unable to reach the same efficacy exhibited by S-14. This activity is consistent with substantial partial agonist activity.

Yeast Data. A novel yeast-based expression system for the characterization of individual peptides played an important role in this study. A distinct advantage of the yeast expression over mammalian systems is the expression of only one receptor and one heterotrimeric G protein. Thus, the properties of the receptor-ligand interaction may be assessed without complication from contaminating receptor subtypes. The rat SST_2, when expressed in yeast, exhibited radioligand binding properties that are similar to those it expressed in mammalian cells and functionally coupled to the yeast pheromone response pathway (31). The yeast expression system provides a functional readout in response to agonist (growth) or antagonist (inhibition of growth) properties of a particular somatostatin analog. This response is manifested by an observable zone of growing yeast cell around the site of the agonist administration. In this manner, agonists of varying affinity and potency may be assayed. Because concentration varies with the square of the radius as it diffuses radially from the site of the application, small changes in the radius of the growth zone correspond to large differences in the affinity of a particular compound for the SST_2. Similarly, the extent to which cells grow within the zone is related to the potency of a particular compound. To assay the effects of compounds with potential antagonist activity, it was necessary to add S-14 (100 nM) to the agar, which stimulated the growth of all cells within the plate. Application of an antagonist blocked the growth-promoting effect of S-14, which yielded a clear zone in an otherwise uniform lawn of growing yeast cells. Because compounds that are toxic would be expected to produce a similar response, all compounds reported herein were tested for growth inhibitory activity that was not receptor-dependent. None of the compounds exhibited toxic effects.

Based on the yeast bioassay, analogs with the L-Cys^2,L-Cys^7 pair (**1** and **3**) were potent agonists (Fig. 3). Analogs with a D-Cys^2,L-Cys^7 pair (**2** and **4**) display potent antagonist properties. The compound with a D-Phe^1 residue (**5**), which seemed to be a partial agonist in the cAMP accumulation assay, seemed to be a weak antagonist in the yeast screen. The data agree well with results obtained from cAMP accumulation studies in mammalian cells.

cAMP versus yeast. The combination of three assays (radioligand binding, cAMP, and yeast bioassay) allows for both quantitative characterization of molecular properties as well as qualitative high-throughput evaluations of the functional properties of large numbers of novel somatostatin ligands. We have used all three assays for the discovery of novel somatostatin antagonists. The radioligand binding assays and cAMP accumulations are well-established quantitative assays. Unfortunately, functional assays, like the

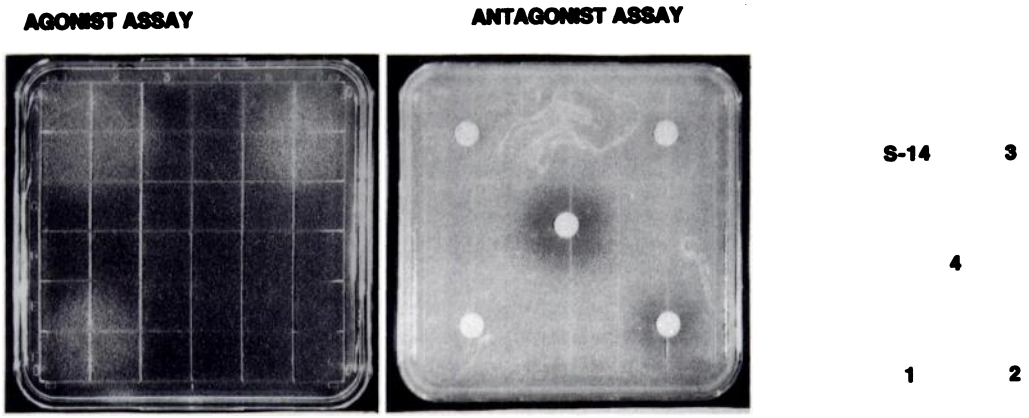

Fig. 3. Yeast-based bioassay of SST ligands. The activity of compounds described herein were assayed in an agar plate bioassay modified from that reported in Price *et al.* (31). Overnight liquid cultures of LY268 [containing the SST_2 (29)] cells in 2 ml of SC medium containing glucose (2%) and lacking tryptophan and uracil were centrifuged, resuspended in 5 ml of SC liquid medium containing galactose (2%), sucrose (0.2%), and lacking tryptophan and uracil, and was incubated overnight at 30°. Cells (2×10^5) were dispersed in 30 ml of SC-galactose (2%), sucrose (0.2%) agar medium lacking tryptophan, uracil, and histidine (adjusted to pH 6.8 with concentrated KOH before autoclaving and equilibrated to 50°) and poured in a 10- × 10-cm sterile Petri plate. For the antagonist assay, S-14 was added to a final concentration of 100 nM before plating. After allowing the agar to harden, 5-μl samples of the designated compounds (1 mM in DMSO) were pipetted onto the surface (agonist assay) or onto sterile filter disks placed on the surface of the agar (antagonist assay). The plates were incubated at 30° for 48 hr. *Right*, identities of the applied compounds.

cAMP accumulation assay, are relatively time consuming and not generally suited for large-scale, rapid screening. The yeast assay complements these traditional assays because it is rapid and provides functional data. A large number of analogs can be screened to identify candidates for further screening in more laborious quantitative assays.

These data are all consistent with the hypothesis that **2** and **4** are selective antagonists of the SST_2 subtype. The key difference between these antagonists and the numerous agonists previously described are the D stereochemistry of Cys^2 and the L stereochemistry of residue 1. In our studies, **4** was the most potent antagonist; the binding affinities were equal to somatostatin but exhibited virtually no residual agonist behavior. The affinity for the receptor is equivalent to somatostatin, and both functional assays, cAMP accumulation and yeast-growth, are consistent with full antagonism of SST_2 subtype. To our knowledge, this is the first full antagonist of somatostatin to be identified. This should provide a valuable tool for studying this receptor *in vitro* and *in vivo*.

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Send reprint requests to: John R. Hadcock, Wyeth-Ayerst Research, CN 8000, Princeton, NJ 08543-8000. E-mail: hadcocj@war.wyeth.com