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A Linear Hexapeptide Somatostatin Antagonist Blocks Somatostatin Activity *In Vitro* and Influences Growth Hormone Release in Rats

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ABSTRACT

Somatostatin (SRIF) is the main inhibitory peptide regulating growth hormone (GH) secretion. It has been difficult to establish the role of endogenous SRIF release in the absence of pure SRIF antagonists. Although several SRIF antagonists have recently been described, none have been shown to possess *in vivo* activity in the absence of added SRIF. Here, an SRIF antagonist with no detectable agonist activity has been identified from a synthetic combinatorial hexapeptide library containing 6.4×10^7 unique peptides. Each peptide in the library is amino-terminally acetylated and carboxyl-terminally amidated and consists entirely of D-amino acids. A SRIF-responsive yeast growth assay was used as a primary screening tool, and cAMP accumulation, competitive binding, and microphysiometry also were used to confirm and further characterize SRIF antagonist activity. The hexapeptide library was screened in stepwise iterative fashion to identify AC-178,335, a pure SRIF antagonist of the sequence Ac-hfirwf-NH_2. This D-hexapeptide bound SRIF receptor type 2 with an affinity constant (K_i) of 172 ± 12 nM, blocked SRIF inhibition of adenylate cyclase *in vitro* (IC_{50} = 5.1 ± 1.4 μM), and induced GH release when given alone (50 μg intravenously) to anesthetized rats with or without pretreatment with a long-acting SRIF agonist.

SRIF, a peptide of 14 or 28 amino acids found in the central nervous system and many peripheral tissues, is a negative regulator of numerous biological processes in animals and is the major known inhibitor of growth hormone secretion. SRIF acts via cell surface receptors that are members of the G protein-linked family of seven-transmembrane domain receptors. Five SRIF receptor subtypes have been described ($SST_{1–5}$; Reisine and Bell, 1995), all of which bind S-14 but vary in their affinity for S-28 and various synthetic SRIF agonists (Raynor *et al*., 1993). SRIF signaling occurs for the most part through inhibitory G proteins, manifesting a variety of physiological changes in the cell, including inhibition of adenylate cyclase, blockade of voltage-dependent Ca^{2+} channels, and activation of potassium channels and tyrosine phosphatase. SST_2 seems to be the major subtype involved in GH secretion and regulation, although many of the other subtypes are present in both arcuate nucleus and pituitary (Gillies, 1997). SRIF also has important sites of action in the central nervous system, pancreas, spleen, adrenals, and gastrointestinal tract (reviewed in Reisine and Bell, 1995).

Since the discovery of SRIF, large numbers of agonist analogs have been synthesized, exhibiting such properties as high affinity, prolonged activity, subtype selectivity, or a combination (Veber *et al*., 1981; Murphy *et al*., 1985; Cai *et al*., 1986). SRIF antagonists, on the other hand, only recently have been reported (Bass *et al*., 1996; Wilkinson *et al*., 1996; Murphy *et al*., 1997), and thus far, no biological activity has been attributed to them *in vivo* in the absence of exogenously added SRIF. Although its direct effects on pituitary GH release are inhibitory, SRIF has been shown to have paradoxically positive actions on GH release *in vivo* (Clark and Robinson, 1988; Tannenbaum *et al*., 1989), so it is difficult to predict the net effects of SRIF antagonism. Nevertheless, SRIF antagonists may have a use in medicine or commercial agriculture. One reason for the difficulty in isolating SRIF antagonists has been the lack of a convenient direct assay because SRIF activity usually is measured *in vitro* as the inhibition of an artificial stimulation. However, a sensitive, positively acting SRIF assay has been recently developed in

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ABBREVIATIONS: SRIF, somatostatin; S-14, somatostatin-14; S-28, somatostatin 28; GH, growth hormone; SST_n, somatostatin receptor subtype, where *n* is the subtype; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HEK, human embryonic kidney; TSH, thyroid-stimulating hormone; GRF, growth hormone-releasing factor; IGF-I, insulin-like growth factor I.

yeast, whereby an SRIF receptor (SST_2) has been functionally linked to the *Saccharomyces cerevisiae* pheromone response pathway, yielding yeast strains that grow in response to SRIF (Price *et al*., 1995). This assay provided a high throughput format to screen for putative SRIF antagonists from complex mixtures of synthetic peptides.

Virtually all synthetic SRIF analogs are cyclic peptides, ranging in size from six to eight amino acids. One notable exception, BIM 23066, is a linear octapeptide that displays some attributes of an SRIF antagonist (Bass *et al*., 1996). Other linear hexamer peptide agonists have also been reported (Raynor *et al*., 1993). Thus, the synthetic combinatorial hexapeptide library chosen for this study had the potential to contain sequences with SRIF antagonist properties. This library contained 6.4×10^7 individual N-acetylated and C-amidated peptides, synthesized entirely from D-isomers of the 19 natural amino acids plus glycine (Houghten *et al*., 1991). Similar libraries have provided the source material for the isolation of several biologically active peptides, such as opioid agonists (Dooley *et al*., 1994), antimicrobial peptides, enzyme inhibitors, and antigenic determinants (Blondelle *et al*., 1995, and references therein). This library consists of 400 samples (each containing 160,000 different peptides) in which the first two amino acids are fully defined (X) and the latter four amino acid positions are represented by a randomized combination of all 20 D-amino acids (O), giving the structure Ac-X-X-O-O-O-O-NH_2. In screening the library, a series of iterative synthetic steps allows the identification of peptide mixtures in which each randomized position is progressively defined, finally resulting in the identification of individual hexapeptide sequences.

In this report, we identify a linear all D-amino acid hexapeptide with *in vitro* properties consistent with a pure SRIF antagonist and provide the first *in vivo* evidence for the action of an SRIF antagonist on GH release. Some of this work has been described in preliminary form (Baumbach *et al*., 1997).

Experimental Procedures

Synthesis of Synthetic Combinatorial Peptide Library, Peptide Mixtures, and Individual Peptides

A synthetic combinatorial peptide library was prepared using methylbenzhydrylamine polystyrene resin and standard t-boc chemistry. The hexapeptide library was prepared essentially as described previously (Houghten *et al*., 1991) using D-enantiomers of the 20 natural L-amino acids. Synthesis proceeded in six steps, in which equimolar mixtures of the 20 D-amino acids were incorporated into positions 3–6. Each of 400 peptide samples contained unique D-amino acid combinations in positions 1 and 2 and were acetylated at the amino terminus, amidated at the carboxyl terminus, and dissolved in water at a concentration of 5 mg/ml. Each of the 400 mixtures contained 160,000 peptides of the form Ac-X-X-O-O-O-O-NH_2, where X represents defined D-amino acids and O represents a random mixture of all 20 D-amino acids.

For each of four subsequent syntheses, called iterations, 20 fresh samples were prepared identically to the original 400 samples, except that progressively more amino acid positions were fully defined. Thus, the first iteration yielded peptides of the structure Ac-X-X-X-O-O-O-NH_2, and each sample contained a mixture of 8000 different peptides. Finally, the fourth iteration yielded pure, fully defined hexamers (Houghten *et al*., 1991). Thus, five different types of samples were produced: the original library, three progressively less complex mixtures (first, second, and third iterations), and pure peptides (fourth iteration). The original library, iterations, and pure peptides were synthesized by Multiple Peptide Systems (San Diego, CA). BIM-23066a, an analog of BIM-23066 in which the first amino acid position is L-Phe instead of D-Phe, was synthesized by J. Chiarello (American Cyanamid, Princeton, NJ); and MK678 and AC-178,335 were synthesized by R. Bass (American Cyanamid, Princeton, NJ). Individual peptides and mixtures were analyzed by mass spectral analysis (ABI Plasma Desorption Mass Spectrometer).

SRIF Antagonist Assays Using *S. cerevisiae* (Yeast) Cells Expressing SSTR2

The yeast strain LY 364 (MATa ura3–52 leu2 his3 trp1 lys2 ade2 far1::LYS2 fus1::FUS1-HIS3 gpal::hisG sst2::ADE2) also houses the plasmids pJH2, containing the rat SST_2 cDNA under the control of the GAL 1/10 promoter, and pLP82, with the rat $G_{\alpha i2}$ gene under the GPA1 promoter (Price *et al*., 1995). For the yeast plate assay, LY364 cells were grown and plated in square (20 × 20 cm) agar petri dishes with SRIF (S-14, 10 nM) as described previously (Price *et al*., 1995). Sterile filter disks were placed on the surface of the agar and saturated with 10 μl of water or dimethylsulfoxide containing 5 mg/ml concentration of test compounds. After 3 days, the plates displayed a uniform cloudy background of LY264 cells growing in response to the added SRIF. The test compounds, which diffused radially through the agar, exhibited SRIF antagonist activity by a clear zone of growth inhibition surrounding the filter disk. This zone was quantified by measuring its diameter (mm), which, for compounds of similar molecular weight, varied according to the potency of the SRIF antagonist. BIM 23066a (displaying similar properties as BIM 23066) was the positive control in this assay. For the yeast proliferation assay, LY364 cells were seeded (200 μl/well, 10^5 cells/ml) onto 96-well trays and grown for 24 hr at 30° (Price *et al*., 1995) in the presence of SRIF (S-14), AC-178–335, or both, after which absorbance (620 nM) was measured with a plate reader.

cAMP Accumulation Assay for Analysis of SRIF Antagonists *In Vitro*

Stimulation of cells. GH_4C_1 (rat pituitary) cells were stably transfected with a plasmid containing a cytomegalovirus-driven rat SST_2 cDNA (Strnad *et al*., 1993). Resulting GH_4C_1/SST_2 cells (clone 20, containing 41 pmol of SST_2/mg of membrane protein; Tentier *et al*., 1997) were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Cells were washed and resuspended in induction buffer [phosphate-buffered saline (1.5 mM KH_2PO_4, 150 mM NaCl, and 2.5 mM $Na_2HPO_4 \cdot 7H_2O$) containing 100 μM 3-isobutyl-1-methylxanthine and 2 mM $CaCl_2$] at 2×10^6/ml. We added 50 μl of stimulants in induction buffer into triplicate wells of a 96-well tray, including (at final concentration) 1.25 μM forskolin, 100 nM SRIF, or 10 nM of the SSTR2-selective agonist MK678 and test compounds. To initiate stimulation, 50 μl (10^5) of cells was added to each well, and the tray was shaken for 10 sec and placed at 37° for 15 min. Stimulation was arrested by lysing cells with 15 μl of 0.33 N HCl/well for 30 min at 37°. Samples were neutralized by the addition of 15 μl of 0.25 N NaOH/50 mM HEPES, pH 7.4.

cAMP assay. Accumulated cAMP in the samples was measured by scintillation proximity assay (SPA; Amersham, Arlington Heights, IL). Each of the following reagents (50 μl) were added per well in a 96-well tray: supernatants from the cell stimulation, ^{125}I-cAMP (5×10^5 cpm), scintillant-impregnated beads conjugated to monkey anti-rabbit IgG antibodies, and rabbit anti-cAMP antibodies. For the standard curve, known amounts of cAMP (0.2–12.8 pmol) were added in place of cell supernatants. The 96-well tray was sealed, shaken at room temperature overnight, and measured for bound radioligand in a MicroBeta liquid scintillation counter (Wallac, Gaithersburg, MD). The standard curve was determined for each experiment by performing a nonlinear regression analysis of cpm measured for the cAMP standards versus their log concentrations,

and test sample values were determined by fitting to the standard curve using Prism software (GraphPAD Software, San Diego, CA).

Competitive binding assays. Tissue culture cell lines expressing transfected cDNA clones (SST_2 in GH_4C_1 and SST_5 in HEK 293 cells) were used as a source of plasma membranes that specifically bind ^{125}I-S-14 (Amersham). Membranes were prepared (Eppler *et al.*, 1992) and binding assays were performed (Carrick *et al.*, 1995) as described previously. Membrane protein (3 and 5 μg) was used per sample for SST_2 and SST_5, respectively. Bound radioligand was determined by counting in a MicroBeta Liquid Scintillation counter (Wallac), and assays were performed in triplicate. Binding data were plotted and IC_{50} and K_i values were calculated for test compounds using Prism software (GraphPAD Software).

Microphysiometry. Real-time analysis of the effects of SRIF agonists and antagonists in GH_4C_1/SST_2 cells was performed with a Cytosensor microphysiometer (Molecular Devices, Sunnyvale, CA). The extracellular acidification rate was measured by silicon microphysiometry essentially as described previously (McConnell *et al.*, 1992). Briefly, GH_4C_1/SST_2 cells were plated 2 days before the experiment in Cytosensor microphysiometer cups at 3×10^5 cells/cup. For the experiment, cups were placed on the Cytosensor and perfused with bicarbonate-free Dulbecco's modified Eagle's medium, pH 7.4, in a repeating 2-min cycle, whereby cells were perfused for 90 sec, followed by 30 sec without perfusion. During the latter phase, the extracellular acidification rate was measured. Cells were equilibrated on the instrument for 1 hr, after which treatments were given for 6 min, and the response (peak acidification rate increase) was measured ~10 min later. Cells reached base-line values 30 min after the start of treatment and then were retreated. For antagonist assays, cells were exposed to peptide mixtures at a concentration of 20 μM or pure peptides at a concentration of 2 μM, simultaneously with 2 nM MK678. Potency (intrinsic activity) was assessed by the percent reduction in the response to MK678 alone.

Animal studies. All animal experiments were conducted with the approval of the Institutional Animal Use and Care Committee. Male Sprague-Dawley rats were housed under temperature-controlled, 12-hr light/dark conditions (light from 6:00 a.m. to 6:00 p.m.) in wire cages and given rat chow and water *ad libitum*. Rats were anaesthetized with Sagatal (60 mg/kg i.p.) and fitted with jugular catheters, allowing intravenous treatment as well as manual blood sampling. Samples of plasma were assayed for rat GH, and in some cases also for rat prolactin and rat TSH, by specific radioimmunoassays using reagents supplied by National Institute of Diabetes and Digestive and Kidney Diseases. Treatment regimens and group size are detailed in text and figure legends.

Peptides used *in vivo* were GRF (hGRF 1–29-NH_2) and the hexapeptide GH secretagogue GHRP-6 (Ferring AB, Malmo, Sweden) and recombinant human IGF-I (Genentech, South San Francisco, CA; courtesy of Ross Clark) and were diluted in heparinized saline immediately before use. Unless otherwise stated, data are given as mean ± standard error, and differences among groups were determined by unpaired *t* tests.

Results

Primary Screening of a Combinatorial Synthetic Peptide Library for SRIF Antagonists

Each of the 400 peptide mixtures in the D-amino acid synthetic library was spotted onto a filter disc resting on an agar plate that contained LY 364 cells and 10 nM S-14. After 3 days, the plates were inspected for zones of inhibition on the lawn of cells growing in response to SRIF. In the first round of screening, where each sample consisted of 160,000 different peptides, most samples failed to inhibit yeast growth. However, faint zones of inhibition were observed around several filter discs. This is shown in the uppermost section of Table 1, in which peptide mixtures are identified by the defined amino acid positions at their amino terminus. Note that BIM 23066a was included as a positive control for SRIF antagonism. Based on these results, as well as on competitive binding data (not shown), two samples were pursued: Trp-Tyr and His-Phe. For each of these, the first iteration entailed the synthesis of 21 new peptide mixtures, all of which shared the same two amino-terminal amino acids: 20 of the samples each contained a different amino acid at the third position, and the 21st was a resynthesis of the original sample. On testing of the first iteration, none of the samples containing Trp-Tyr at the amino terminus displayed detectable activity, and this combination was abandoned. Samples containing His-Phe, on the other hand, proved to be active, as shown in the second section of Table 1.

In these experiments, slight changes in the growth characteristics, growth conditions, and plating density of the LY 364 cells resulted in somewhat variable plate turbidity between tests (quantitative measurement of the zones was performed by three independent readers). Thus, the nominal sizes of inhibition zones should be directly compared only within each test. However, the trend toward larger zones is apparent (e.g., in comparison with BIM 23066a) as the peptide mixtures become less heterogeneous and hence more potent. The sensitivity and facility of the yeast antagonist assay were essential for the determination of active samples during the library and first iteration screening steps. In later rounds of screening (second through fourth iterations), the low sample number and increasing sample purity allowed the additional use of other, less sensitive assays (i.e., competitive binding, cAMP accumulation, and microphysiometry; not shown). With few exceptions, these other SRIF antagonist assays, as well as the competitive binding assay, supported the rank order of potency among peptide mixtures as measured by the yeast SRIF antagonist assay. On analysis of each iteration, a single amino acid was chosen for that position in the peptide. If the yeast assay did not clearly identify a single lead sample, data from the other assays were considered. For example, in the fourth iteration, several peptides showed similarly potent activity in the yeast assay. The peptides ending in D-Met and D-Phe had IC_{50} values of 932 and 366 nM (competitive binding at SST_2) and had intrinsic activities of 32% and 52% (microphysiometry), respectively, indicating that despite similar scores in the yeast assay, the peptide with D-Phe in this position was the more potent antagonist. In addition, D-Trp in this position gave an IC_{50} value of 431 nM and an intrinsic activity of 39.6%, suggesting that it also has better antagonist characteristics than the D-Met peptide. Thus, after four iterative rounds of testing, the peptide highlighted in the bottom section of Table 1 was chosen as the most potent SRIF antagonist. The structure of this peptide, denoted AC-178,335, is acetyl-D-His-D-Phe-D-Ile-D-Arg-D-Trp-D-Phe-NH_2.

Synthesis and Testing of Analogs of AC-178,335

A series of analogs were synthesized based on the structure of AC-178,335 to determine which amino acid positions are important for antagonist activity and binding affinity. These analogs include replacement of each position by D-alanine (alanine scan), deletion of each position (deletion scan), replacement of each position by the corresponding L-amino acid (L-amino acid scan), specific substitutions based on perfor-

mance of the peptide mixtures during the course of the iterative process, frame shifting of amino acid order, transposition of amino acids, and cyclization of the peptide. The assays that were performed were the yeast plate assay (size of zone of inhibition in mm), the GH_4C_1/SST_2 competitive binding assay (K_i, μM), and the GH_4C_1/SST_2 cAMP accumulation assay (percent reversal of SRIF activity at 2 mM peptide concentration in the presence of 1.25 mM forskolin and 10 nM concentration of the SRIF superagonist MK678).

Data from the testing of this analog series are shown in Table 2, in which the structure of each analog is given using the single-letter amino acid code (lowercase denotes the D-stereoisomer and uppercase denotes the L-stereoisomer). Although a few analogs with conservative changes displayed no loss of binding at SST_2, none of the analogs possessed SRIF antagonist activity as potent as that of the parent AC-178,335. This suggested that the iterative process used in the isolation of AC-178,335 was an effective strategy for the identification of the most potent single peptide in a complex mixture based on biological activity. The alanine scan indicates that only the third position (D-Ile) can be substituted without significant loss of activity. Surprisingly, at least three different positions can be eliminated, yielding pentamers, without completely losing activity in the yeast screen. Single substitutions of L-amino acids can be accommodated at every position except for the third (Ile). In substituting amino acids at positions where they showed activity during the early screening steps, it seems that only relatively conservative changes can be accommodated while retaining activity: Tyr or Trp for Phe at position 2; Val for Ile at position 3; and Lys for Arg at position 4. Transposition, frame shifting, and cyclization all yielded peptides with lower antagonist activity.

Pharmacological Profile of AC-178,335

Competitive binding. Among the limited number of analogs that were tested, AC-178,335 retained the best SRIF antagonist characteristics and thus was characterized further *in vitro*. Competitive binding assays were performed using membranes isolated from GH_4C_1/SST_2 and HEK 293/

TABLE 1
Screening of a combinatorial synthetic peptide library using the yeast SRIF antagonist bioassay

Sample type	AA_1	AA_2	AA_3	AA_4	AA_5	AA_6	Zone
							mm
Library	His	Tyr	Ran	Ran	Ran	Ran	12.5
	His	Trp	Ran	Ran	Ran	Ran	12
	His	**Phe**	Ran	Ran	Ran	Ran	**13**
	Trp	Tyr	Ran	Ran	Ran	Ran	12.5
	Phe	His	Ran	Ran	Ran	Ran	13.5
BIM-23066a							19.5
1st iteration	His	Phe	Ran	Ran	Ran	Ran	5
	His	Phe	Cys	Ran	Ran	Ran	9
	His	Phe	Val	Ran	Ran	Ran	9.5
	His	Phe	Leu	Ran	Ran	Ran	7
	His	Phe	Thr	Ran	Ran	Ran	7
	His	**Phe**	**Ile**	Ran	Ran	Ran	**10**
	His	Phe	Trp	Ran	Ran	Ran	7
BIM-23066a							13
2nd iteration	His	Phe	Ile	Ran	Ran	Ran	11
	His	Phe	Ile	His	Ran	Ran	11
	His	Phe	Ile	Pro	Ran	Ran	11.5
	His	**Phe**	**Ile**	**Arg**	Ran	Ran	**12.5**
	His	Phe	Ile	Asn	Ran	Ran	11
	His	Phe	Ile	Trp	Ran	Ran	11
	His	Phe	Ile	Lys	Ran	Ran	12.5
BIM-23066a							11
3rd iteration	His	Phe	Ile	Arg	Ran	Ran	15
	His	Phe	Ile	Arg	His	Ran	12
	His	Phe	Ile	Arg	Leu	Ran	12
	His	Phe	Ile	Arg	Arg	Ran	13
	His	Phe	Ile	Arg	Tyr	Ran	15
	His	Phe	Ile	Arg	Ile	Ran	12
	His	Phe	Ile	Arg	Met	Ran	13
	His	**Phe**	**Ile**	**Arg**	**Trp**	Ran	**23**
	His	Phe	Ile	Arg	Gly	Ran	18
BIM-23066a							13
4th iteration	His	Phe	Ile	Arg	Trp	Ran	15
	His	Phe	Ile	Arg	Trp	Leu	15
	His	Phe	Ile	Arg	Trp	Thr	14
	His	Phe	Ile	Arg	Trp	Tyr	16
	His	Phe	Ile	Arg	Trp	Ile	16
	His	Phe	Ile	Arg	Trp	Ala	15
	His	Phe	Ile	Arg	Trp	Asn	15
	His	Phe	Ile	Arg	Trp	Met	18
	His	Phe	Ile	Arg	Trp	Trp	16
	His	**Phe**	**Ile**	**Arg**	**Trp**	**Phe**	**18**
	His	Phe	Ile	Arg	Trp	Gly	13
	His	Phe	Ile	Arg	Trp	Gln	16

Samples in bold type were chosen for further synthesis. Boxed regions of the table denote individual assays. BIM-23066a was tested at 1 mM in DMSO. Ran, random; zone, inhibition zone.

SST_5 cells. In both assays, the radioligand ^{125}I-S-14 was competed with test peptides. For SST_2, S-14 and AC-178,335 were the competitive ligands, whereas for SST_5, S-28 (its endogenous high affinity ligand) was used. As shown in Fig. 1, AC-178,335 competes specifically with ^{125}I-S-14 for binding to SST_2 and SST_5, although with much lower affinities than the endogenous ligands (S-14 and S-28, respectively). Subtype selectivity of AC-178,335 for these two receptors is modest, with the K_i value being ~160 nM for SST_2 and ~230 nM for SST_5. Saturation binding experiments also suggest that AC-178,335 competes with S-14 at SST_2 (data not shown). The mean K_i value for AC-178,335 at SST_2 is 172 ± 12 nM (five experiments).

cAMP accumulation. The rat pituitary cell line GH_4C_1 expresses endogenous SST_2 (Eppler *et al.*, 1992) whose function can be measured by inhibition of artificially (forskolin) induced cAMP levels (Koch and Schonbrunn, 1984). GH_4C_1/SST_2 cells also carry an exogenous transfected rat SST_2 cDNA (Hipkin *et al.*, 1997), conferring 100-fold higher SST_2 levels than wild-type GH_4C_1 cells (clone 20; 41 pmol/mg membrane protein; Tentier *et al.*, 1997). This cell line provides a robust system with a wide dynamic range for studying the action of SRIF antagonists and is particularly useful in characterizing compounds with low agonist activity that is undetectable using wild-type GH_4C_1 cells or other assay systems. Fig. 2A depicts an experiment in which cAMP levels are measured in forskolin-stimulated cells with increasing doses of either S-14 or the superagonist MK678 in the presence or absence of 10 mM AC-178,335. The relationships of the curves suggest that AC-178,335 is directly competing with the SRIF agonist binding sites. Fig. 3 illustrates an experiment in which GH_4C_1/SST_2 cells were treated with 1.25 μM forskolin along with varying concentrations of AC-178,335. The lack of cAMP inhibition displayed in the presence of high concentrations of AC-178,335 confirms its lack of agonist activity. Antagonist activity of AC-178,335 is also shown in Fig. 3, in which increasing concentrations of AC-178,335 completely reversed the effects of the SRIF agonist MK678, with an IC_{50} value of 4.5 μM (mean IC_{50} = 5.1 ± 1.4 μM, three experiments). Figs. 2A and 3 also show that with high concentrations of AC-178,335, cAMP levels tended to be higher than in the presence of forskolin alone.

TABLE 2
SRIF antagonist activity of AC-178,335 analogs
Analog structure is given in single letter amino acid code, with lower case denoting D-amino acids and upper case denoting L-amino acids. All peptides are amidated at the carboxyl terminus and acetylated at the amino terminus.

Analogs	Yeast assay[a]	GH_4C_1/SST_2 antagonist[b]	Binding (SST_2)[c]
1. h-f-i-r-w-f	20	85.2	0.16
Alanine scan			
2. a-f-i-r-w-f	None	13.0	11.8
3. h-a-i-r-w-f	None	9.33	6.6
4. h-f-a-r-w-f	16	41.8	0.75
5. h-f-i-a-w-f	10	44.1	1.35
6. h-f-i-r-a-f	9	13.4	6.55
Deletion scan			
7. f-i-r-w-f	None	−7.2	29.8
8. h-i-r-w-f	None	2.76	22.8
9. h-f-r-w-f	9	6.36	2.4
10. h-f-i-w-f	14	25.3	0.95
11. h-f-i-r-f	14	12.1	2.05
12. h-f-i-r-w	16	29.1	0.47
L-Amino acid scan			
13. H-f-i-r-w-f	15	26.2	1.42
14. h-F-i-r-w-f	16	29.6	0.95
15. h-f-I-r-w-f	9	9.71	4.6
16. h-f-i-R-w-f	17	43.6	0.75
17. h-f-i-r-W-f	19	50.2	0.7
18. h-f-i-r-w-F	19	46.7	0.85
Positions 1–5 Alternative choices during iterations			
19. k-y-i-r-w-f	9	33.3	1.27
20. h-y-i-r-w-f	18	56.5	0.27
21. h-w-i-r-w-f	18	54.7	0.32
22. W-h-i-r-w-f	None	27.4	4.9
23. w-y-i-r-w-f	None	5.64	1.35
24. f-h-i-r-w-f	None	65.2	9.5
25. f-r-i-r-w-f	None	10.8	9.0
26. h-f-i-r-g-f	9	4.51	14.0
27. h-f-i-k-w-f	20.5	71.2	0.17
28. h-f-v-r-w-f	20	71.2	0.14
Transposed positions and frame shifted peptides			
29. h-*w*-i-r-*f*-f		21.3 (100μM)	0.97
30. h-f-*w*-r-*i*-f		12.9 (100μM)	2.8
31. h-f-*r*-*i*-w-f		13 (100μM)	2.5
32. w-f-h-f-i-r		−6 (100μM)	37.2
33. i-r-w-f-h-f		11.5 (100μM)	2.8
34. C-h-f-i-r-w-f-C		9 (10 μM)	1.95
(cyclic)		25.9 (100μM)	
35. h-f-i-r-w-f		21.5 (10 μM)	0.14
(cyclic)		122 (100μM)	

[a] Yeast assay data are diameter of inhibition zone (mm); 10 pmol of each peptide was tested in the yeast assay.
[b] The GH_4C_1/SST_2 antagonist assay data are intrinsic activity (percent reversal of 10 nM MK678 inhibition of forskolin-stimulated cAMP levels); peptides were tested at 2 μM except as indicated.
[c] The GH_4C_1/SST_2 binding assay measured competition with ^{125}I-S-14; values are K_i in μM.

Yeast growth assay. The yeast strain LY364, expressing rat SST_2, proliferates in response to SRIF. To further assess the ability of AC-178,335 to block SRIF function, a yeast growth assay was developed in liquid culture. This assay enabled effective and inhibitory concentrations to be measured in yeast. Fig. 2B illustrates such an assay, in which the S-14 response is measured as absorbance of growing yeast cells. In this system, the EC_{50} value for S-14 is 94 nM, and for S-14 in the presence of 10 μM AC-178,335, the EC_{50} value is 1135 nM. These values contrast with those obtained in the cAMP assay shown in Fig. 2A, where the EC_{50} value is 6.2 nM for S-14 and 340 nM for S-14 in the presence of 10 μM AC-178,335. The IC_{50} value for AC-178,335 in the yeast growth assay is 561 nM when measured against 100 nM S-14.

AC-178,335 in Anesthetized Rats

The above results showed that AC-178,335 was able to antagonize the effects of exogenous SRIF agonists *in vitro*.

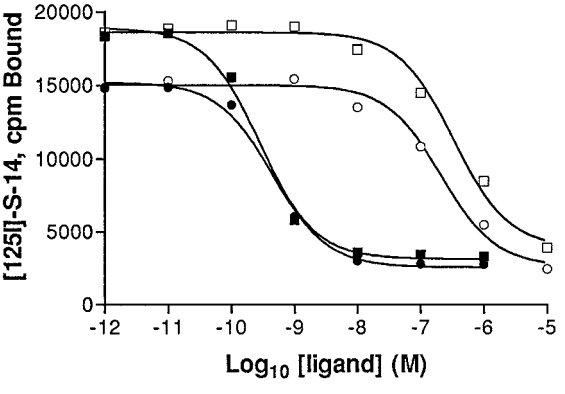

Fig. 1. Competition binding of AC-178,335 with ^{125}I-S-14 in GH_4C_1/SST_2 and HEK 293/SST_5 membranes. S-14 (●) and AC-178,335 (○) were competed with ^{125}I-S-14 in GH_4C_1/SST_2 membranes. S-28 (■) and AC-178,335 (□) were competed with ^{125}I-S-14 in HEK 293/SST_5 membranes. Representative of three experiments. K_i value for S-14 at SST_2 is 0.27 nM; K_i value for S-28 at SST_5 is 0.17 nM.

This peptide therefore was tested *in vivo* to explore the possibility that AC-178,335 could antagonize both endogenous and exogenous SRIF activities.

Anesthetized male rats show constant basal GH secretion that is sensitive to increase and decrease by GRF and SRIF administration, respectively. Groups of rats were given 5 μg (four experiments), 25 μg (four experiments), or 50 μg (six experiments) AC-178,335 by intravenous injections causing a brief, dose-dependent release of GH into the bloodstream (Fig. 4A). At 60 min later, 10 μg of the long-acting SRIF agonist BIM-230^{14}C (Painson and Tannenbaum, 1991) was given to suppress basal GH secretion. A further injection of AC-178,335 (50 μg) reversed this inhibition and induced a larger rise in blood GH levels. Control experiments were performed under the same conditions, with two analogs of a similar or identical amino acid content to that of AC-178,335 but with much reduced SRIF antagonist activity *in vitro* (peptides 22 and 32; Table 2). These peptides had no effect on GH secretion in the same assay paradigm (Fig. 4B), suggesting that the effects seen by AC-178,335 were sequence specific and related to its SRIF antagonist characteristics *in vitro*. We found no evidence for any partial SRIF agonist activity of AC-178,335, and its antagonist activity was specific for growth hormone. In other experiments, AC-178,335 again reversed the suppression of GH by the SRIF agonist BIM-230^{14}C but had no effect on prolactin or TSH levels measured in the same samples (Table 3).

AC-178,335 was tested in combination with other factors known to release GH in the rat. Fig. 5 shows the results of two experiments in which groups of anesthetized rats (six experiments) were given a maximally effective dose of GRF (1 μg) alone or in combination with AC-178,335 (50 μg). GH levels in the rats treated with GRF + AC-178,335 rose faster and reached higher initial peak values than in animals treated with GRF alone, suggesting that AC-178,335 was not

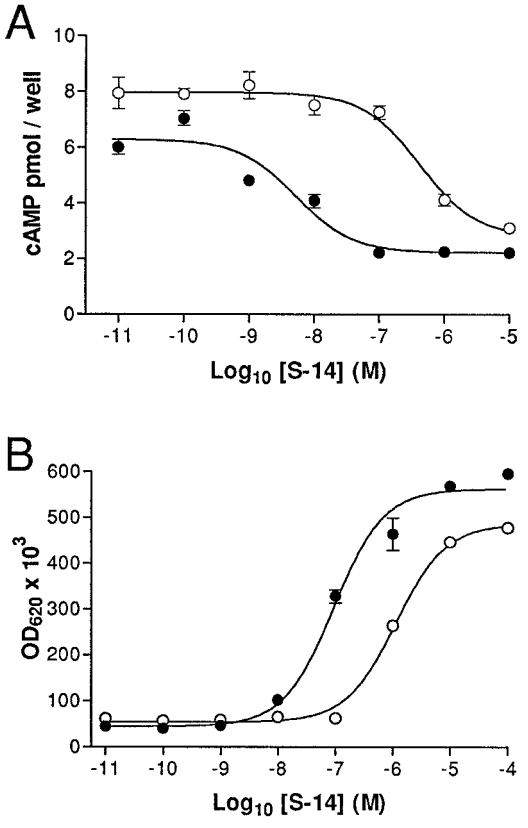

Fig. 2. The effect of 10 μM AC-178,335 on the S-14 dose response at SST_2. A, cAMP accumulation in GH_4C_1/SST_2 cells. B, Growth in liquid culture of yeast strain LY 364. ●, S-14 alone. ○, S-14 + 10 μM AC-178,335.

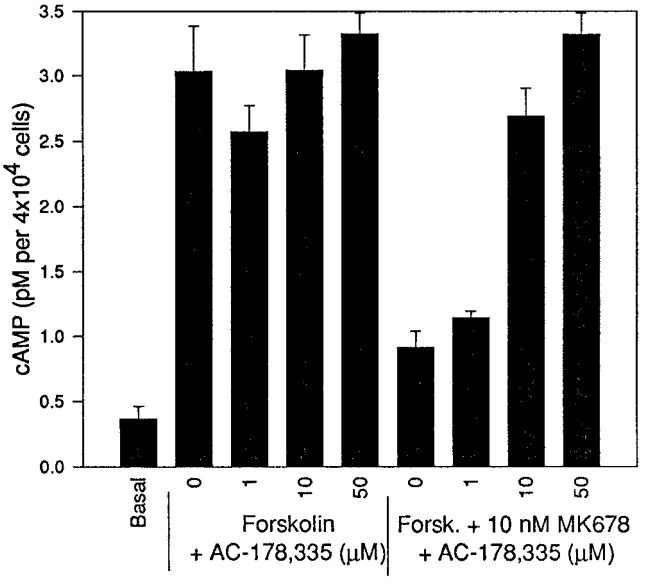

Fig. 3. cAMP accumulation in GH_4C_1/SST_2 cells. For agonist assays, cells are treated with 1.25 μM forskolin and AC-178,335 at concentrations shown; for antagonist assays, 1.25 μM forskolin, 10 nM MK678, and AC-178,335 (at given concentrations) are included in each sample. *F*, Forskolin.

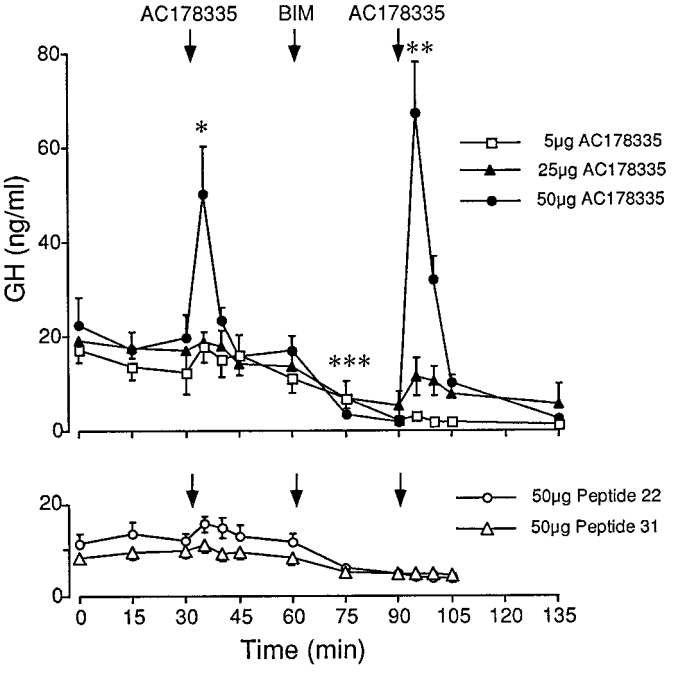

Fig. 4. GH levels in anesthetized rats. *Top*, AC-178,335 is given intravenously at 30 min, at doses shown in legend; at 60 min, 10 μg of BIM-230^{14}C is given; and at 90 min, a second dose of AC-178,335 is given. *Bottom*, the same treatment regimen is used except peptides 22 and 31 are used instead of AC-178,335. Blood is sampled at times indicated and assayed for GH content. *BIM*, BIM-230^{14}C. *, $p < 0.02$ for GH rise at 50 μg of AC-178,335. **, $p < 0.0001$ for GH rise at 50 μg of AC-178,335. ***, $p < 0.0001$ for GH suppression by BIM-230^{14}C.

acting via the same mechanism as GRF and that it could acutely antagonize the effects of endogenous SRIF on the GH response to GRF, although the effect observed was small and short lasting (Fig. 5A). Fig. 5B illustrates the same effect after GH suppression with an exogenous SRIF agonist (BIM-230^{14}C).

Other groups of rats were infused for 2 hr either with the hexapeptide GH secretagogue GHRP-6 (100 μg/hr, seven experiments) to desensitize them to this GH induction pathway or with IGF-I (30 μg/hr, seven experiments), which inhibits GH synthesis and release and may act in part by increasing endogenous SRIF release. During these infusions, an intravenous injection of AC-178,335 again induced similar small increases in blood GH levels, suggesting that the effects of acute SRIF antagonism are not blocked by GHRP-6 desensitization (Fig. 6A), nor are they enhanced by IGF-1 infusions (Fig. 6B).

The foregoing effects of AC-178,335 injections were relatively brief, possibly because of the rapid clearance of the small hexapeptide. To test whether continuous exposure to an SRIF antagonist would be more effective in enhancing GH output in anesthetized rats, AC-178,355 was infused (300 μg/hr) for 2 hr, during which intravenous injections were given of either GRF (1 μg, seven experiments or GHRP-6 (10 μg, seven experiments). Control animals received saline infusions, followed by GRF (four experiments) or GHRP-6 (three experiments) injections. Both GHRP-6 (Fig. 7A) and GRF (Fig. 7B) induced large rises in GH, but these were not significantly altered in animals given a continuous infusion of AC-178,335. This dose of AC-178,335 via infusion (300 μg/hr) is sufficient to give a clear *in vivo* response (data not shown; Baumbach *et al*., 1997).

Discussion

SRIF inhibits a variety of systems, including the secretion of hormones such as GH, glucagon, insulin, and gastrin. High affinity long-acting SRIF agonists such as octreotide have been widely tested and found useful to block the excess GH secretion in acromegaly and to slow the growth of hormone-dependent tumors (Lambert *et al*., 1991). Although not yet shown, it is possible that therapeutic benefits in medicine or commercial benefits in agriculture could arise from the ability to block the inhibitory effects of SRIF. Equally important, the use of pure antagonists in model systems should provide insight into the biological functions of SRIF, including its role in the generation of pulsatile GH secretion.

In the past few years, work from several laboratories has illuminated the path toward biologically active SRIF antagonists. The most promising leads have been high affinity, subtype-selective linear octamers that displayed low *in vitro* activity (Raynor *et al*., 1993). The first of these, BIM-23056, displayed antagonist activity with SST_5 (Wilkinson *et al*., 1996). The other, BIM-23066, bound most tightly to SST_2 and SST_3. A cyclic octameric descendant of BIM-23066, in which the disulfide-linked cysteine pair consisted of one D- and one L-stereoisomer, was shown to antagonize the SRIF response at SST_2 and SST_5 (Bass *et al*., 1996). Agonist activity was not thoroughly tested in the former study, whereas modest agonist activity was reported in the latter (Bass *et al*., 1996). Neither of these antagonists have been demonstrated to have *in vivo* activity in the absence of added exogenous SRIF. This also is the case for other analogs (Murphy *et al*., 1997) that were based on structures reported by Bass *et al*. (1996).

Two recent technical advances encouraged us to attempt to identify novel peptide SRIF antagonists without reference or predisposition to known SRIF analogs. The first of these was a yeast-based SRIF assay (Price *et al*., 1995) with two major advantages over conventional assays: its direct measurement of SRIF activity (i.e., growth in response to SRIF), allowing extraordinary sensitivity in detecting SRIF antagonists by zones of growth inhibition; and its ease of use in testing large sample numbers. The second advance was the development of large combinatorial collections of synthetic peptides in a format that allowed direct testing in a functionally responsive system (Houghten *et al*., 1991; Dooley *et al*., 1994). This was crucial because indirect assays such as competition binding are prone to artifacts and do not easily distinguish agonist from antagonist activity. In the current study, a library containing every possible combination of hexameric, all D-amino acid peptides (3.5×10^7) was tested, and a single peptide, AC-178,335, was subsequently identified in five iterative screening steps using the yeast cell screening assay. Although alternative strategies exist for screening this combinatorial hexapeptide library, such as defining different amino acid positions or proceeding with the iterations in a different order, there is no evidence suggesting that other strategies would lead to a different result. It nevertheless is possible that more potent SRIF antagonists than AC-178,335 reside undetected in the combinatorial library used herein.

The sequence of AC-178,335 (Ac-His-Phe-Ile-Arg-Trp-Phe-NH_2, all D-amino acids), especially considering its essential amino-terminal D-His residue, is clearly distinct from the many SRIF analogs found in the literature (e.g., Veber *et al*., 1981; Murphy *et al*., 1985; Cai *et al*., 1986; Raynor *et al*., 1993). However, its amino acid content is nevertheless reminiscent of synthetic SRIF analogs (Raynor *et al*., 1993). It is particularly interesting that positions 2–5 of AC-178,335 are similar to a portion (positions 7–11) of S-14 itself (Ala-Gly-c[Cys-Lys-Asn-Phe-Phe^7-Trp-Lys-Thr-Phe^{11}-Thr-Ser-Cys]) but in reverse order. Thus, AC-178,335 has, in part, the reverse enantiomeric (retro-inverso) structure of S-14. Retro-in-

TABLE 3
Effects of AC-178,335 on blood levels (ng/ml) of GH, prolactin, and TSH in rats pretreated with SRIF

	Basal[a]	30 min[b]	35 min	40 min	45 min
GH	14.6 ± 5.0	4.4 ± 0.6[c]	29.2 ± 5.3[d]	15.1 ± 3.6	7.8 ± 1.1
Prolactin	21.5 ± 8.1	11.2 ± 5.2	11.3 ± 5.6	8.0 ± 3.9	5.8 ± 2.9
TSH	0.08 ± 0.03	0.13 ± 0.04	0.099 ± 0.032	0.07 ± 0.02	0.11 ± 0.01

[a] 10 μg SRIF agonist (BIM 23014C) is given at 0 time.
[b] AC-178,335 (50 μg) is given immediately after 30-min blood sampling.
[c] $p < 0.01$ versus basal.
[d] $p < 0.01$ versus 30 min.

verso peptide structures have been studied widely for their mimicry of the original peptide, for binding purposes as well as for antigenicity (Guichard *et al.*, 1994), and this also is true for SRIF mimics (Murphy *et al.*, 1985).

The potency of AC-178,335 is relatively low but seemed to represent the best member of the library chosen; a few related analogs of AC-178,335 were synthesized and tested but displayed no improvement in their affinity for SST_2. Surprisingly, however, three of the six pentamers that were tested showed antagonist activity at SST_2. SRIF receptor binding analogs smaller than hexamers are quite uncommon (Veber *et al.*, 1981; Raynor *et al.*, 1993), so our detection of SRIF antagonist activity in pentamers lends hope that smaller molecular weight compounds with potential for oral antagonist activity may be discovered. Further analog work also may reveal higher affinity antagonists with similar characteristics as AC-178,335.

AC-178,335 displayed the intriguing property of increasing forskolin-stimulated cAMP levels *in vitro*, especially at high concentrations. This is reminiscent of a recently described activity of certain β-adrenergic antagonists, which not only lack agonist activity but also reduce the intrinsic constitutive activity of the unliganded receptor (Bond *et al.*, 1995). These compounds, called inverse agonists, are not easily tested because inverse agonists are normally indistinguishable from pure antagonists, and constitutive receptor activity is observed only under conditions of extremely high receptor expression. This condition is met by the GH_4C_1/SST_2 cells used herein (Hipkin *et al.*, 1997; Tentier *et al.*, 1997) and may explain why increased cAMP levels were observed in response to AC-178,335. One way to test this would require a pure neutral antagonist with which to compete the putative inverse agonist, but this may be difficult because other SRIF antagonists reported to date have not been rigorously tested for agonist activity at high concentrations. Alternatively, the development of a constitutively active SRIF receptor, similar to that reported for β-adrenergic receptors (Bond *et al.*, 1995), would allow the potential inverse agonist activity of AC-178,335 to be tested.

Among the many varied actions of SRIF at five different receptor subtypes, its effect on GH synthesis and release is perhaps most widely known. It is thought that the actions of SRIF on GH synthesis and release occur primarily through interactions with SST_2 (Raynor *et al.*, 1993; Beaudet *et al.*, 1995). In this capacity, SRIF seems to perform a dual role: opposing the action of GRF by inhibiting GH release during periods of high SRIF tone (Tannenbaum, 1988) and paradoxically inducing peaks of GH secretion on SRIF withdrawal

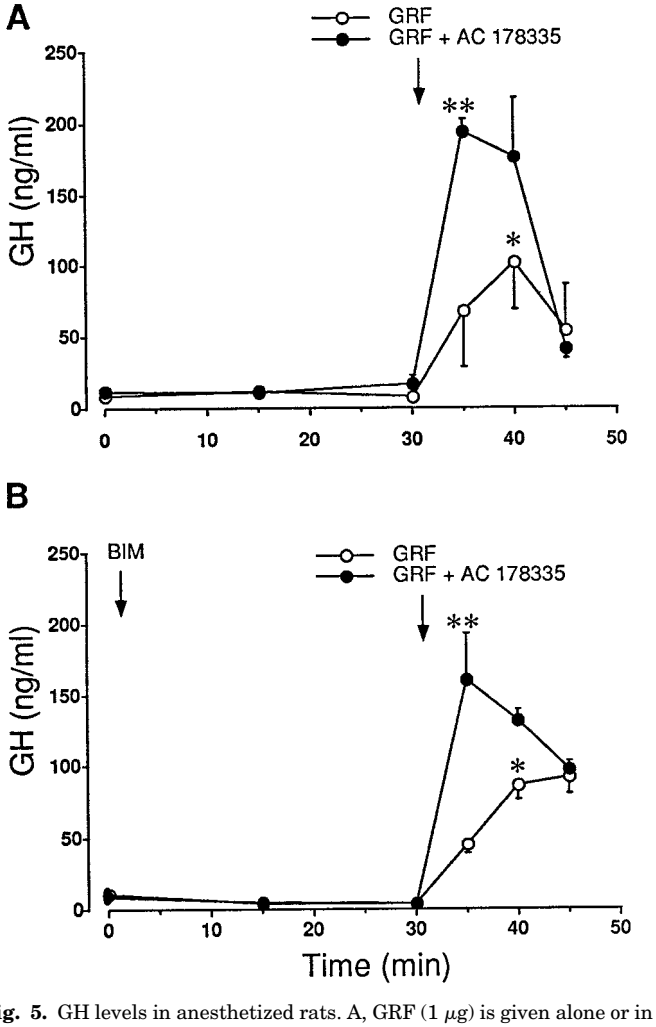

Fig. 5. GH levels in anesthetized rats. A, GRF (1 μg) is given alone or in combination with AC-178,335 (50 μg). Blood was sampled at times indicated and assayed for GH content. *, $p < 0.0001$ for GH rise with GRF alone. **, $p < 0.01$ for GH rise with GRF + AC-178,335 versus GRF alone. B, Same as A, except rats are also pretreated with BIM-230^{14}C (10 μg). *, $p < 0.001$ for GH rise with GRF alone. **, $p < 0.01$ for GH rise with GRF + AC-178,335 versus GRF alone.

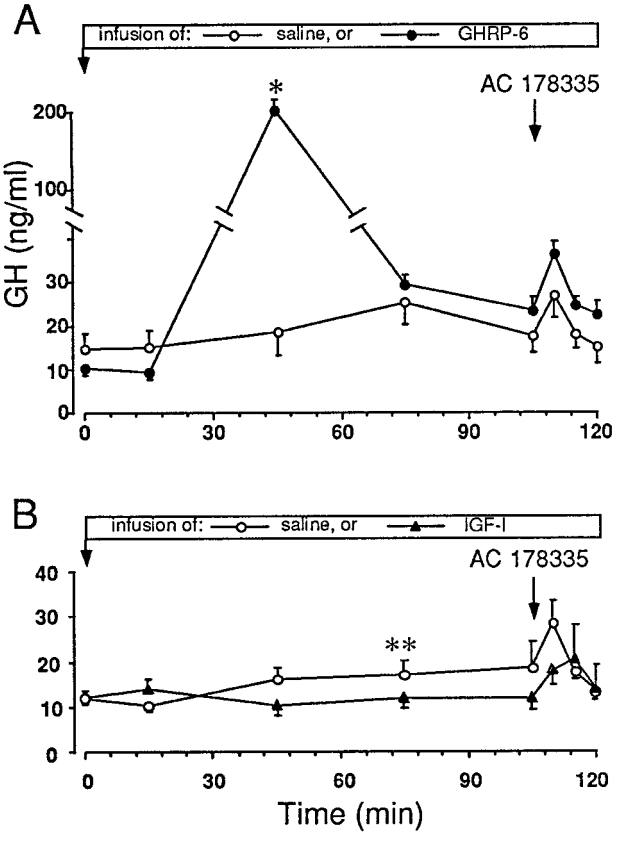

Fig. 6. GH levels in anesthetized rats with constant infusions. Rats were infused with (A) saline or GHRP-6 and (B) saline or IGF-I during the entire 2-hr test period. AC-178,335 (50 μg) was given intravenously at 105 min. Blood was sampled at times indicated and assayed for GH content. *, $p < 0.0001$ for GH rise with GRHP-6. **, $p < 0.02$ for IGF-I infusion versus saline infusion at 75 min.

from the hypothalamic portal system (Clark *et al.*, 1988; Clark and Robinson, 1988). The mechanisms of action of SRIF in controlling GH secretion are complex and are exerted both at the pituitary and the hypothalamus, where SRIF binding sites and receptor gene expression have been demonstrated (Reisine and Bell, 1995). Although the effects of SRIF are predominantly to inhibit GH release, we argued that the acute inhibitory response to SRIF may actually facilitate pulsatile GH release, preventing basal release between pulses and maximizing the amount of GH available for release in response to GRF (Clark *et al.*, 1988), whereas Turner and Tannenbaum (1995) have shown that SRIF exposure prevents down-regulation of the pituitary response to GRF. An effect of an SRIF antagonist on GH pulsatility thus may be not unexpected (Baumbach *et al.*, 1997).

To date, the only *in vivo* studies interrupting SRIF action have involved either immunoneutralization with anti-SRIF antisera (Thomas *et al.*, 1985) or depletion of SRIF with cysteamine (Tannenbaum *et al.*, 1990). These interventions primarily cause an increase in basal GH secretion. We therefore were interested in testing the effects of this SRIF antagonist on GH secretion *in vivo* and to investigate its interactions with other GH secretagogues. We chose to use anesthetized male rats for the *in vivo* studies because, unlike conscious rats, plasma levels of GH are stable and measurable, and anesthesia and surgery are thought to enhance endogenous SRIF secretion. Agonist activity might be revealed by a suppression of basal secretion, whereas pure antagonist activity might be expected to increase GH release.

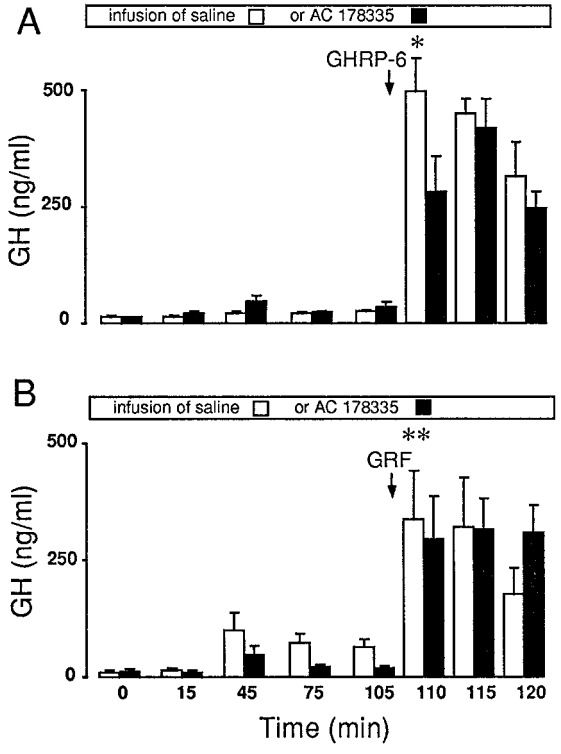

Fig. 7. GH levels in anesthetized rats with constant infusions. Rats infused during the entire test period with saline (*white bars*) or AC-178,335 (*black bars*) were given (A) GHRP-6 or (B) GRF at 105 min. Blood was sampled at times indicated and assayed for GH content. *, $p < 0.01$ for GH rise on GHRP-6 treatment with saline or AC-178,335 infusion. **, $p < 0.05$ and $p < 0.01$ for GH rise on GRF treatment with saline or AC-178,335 infusions, respectively.

AC-178,335 produced brief but reproducible increases in GH release in this model when given alone and were effective in reversing the suppression of basal GH release by prior administration of a long-acting SRIF agonist. As expected from the *in vitro* potency estimates, the *in vivo* effects required relatively large doses of AC-178,335 but were dose dependent, not seen with peptides of similar structure, and were not due to a nonspecific excitation of pituitary cells because prolactin and TSH release in the same rats did not change. Acute AC-178,335 injection also enhanced the effects of a maximal dose of GRF in this model, although the most obvious difference was in kinetics, with plasma GH peak values reached more rapidly when GRF and AC-178,335 were administered together. Again, this effect was seen regardless of whether exogenous SRIF was introduced. The magnitude of GH rise was much smaller than that seen with the direct secretagogues GRF or GHRP-6 and similar to the effects reported by others with SRIF antiserum. A similar, small GH release also can be evoked in anesthetized rats after the withdrawal of exogenous SRIF (Clark *et al.*, 1988). Taken together, these data are consistent with the idea that in anesthetized rats, GH release is under some tonic inhibition by endogenous SRIF, and that AC-178,335 is effective *in vivo* to block endogenous SRIF, evoking a mild rebound release of GH.

Although the evidence from *in vitro* studies pointed to a specific antagonism of SRIF receptors by AC-178,335 as the mechanism of *in vivo* GH release, we did consider other possibilities. For instance, the composition of AC-178,335 does bear some superficial resemblance to the hexapeptide GH secretagogue GHRP-6 (HwAWfK-NH_2), and the variety of structures that mimic the activity of this class of peptides is quite broad (Cheng *et al.*, 1993; Elias *et al.*, 1995). GHRP-6 does synergize with GRF, and it has even been claimed to functionally antagonize SRIF at the pituitary (Bowers *et al.*, 1984). Without a potent and specific GHRP-6 antagonist, it is difficult to exclude the possibility that AC-178,335 somehow mimics GHRP-6 *in vivo*. However, GHRP-6 responses can be desensitized by continuous infusions of a high dose of peptide, leaving the GH responsiveness to GRF intact (Clark *et al.*, 1989). Because AC-178,335 was capable of stimulating GH release in anaesthetized animals desensitized to GHRP-6 and GHRP-6 released GH in animals infused with AC-178,335, it was unlikely that AC-178,335 acted as an agonist for this recently cloned secretagogue receptor (Howard *et al.*, 1996).

We also considered ways of increasing endogenous SRIF secretion to attempt to enhance the effect of AC-178,335. IGF-I blocks GH release in rats and may enhance output of SRIF (Aguila *et al.*, 1993). The GH-releasing effect of AC-178,335 was not enhanced in animals infused with IGF-I, although this result may be confounded by the inhibition of GH synthesis and release, induced by IGF-I acting at the pituitary (Yamashita and Melmed, 1986).

The successful development of AC-178,335 demonstrates the use of screening combinatorial libraries with appropriate *in vitro* screening tools to generate compounds with novel *in vivo* activity. These results provide the first data on a pure SRIF antagonist without agonist activity, which is active *in vivo* and *in vitro*. However, the effects on GH are small and transient, and much further analog development work will be needed to improve potency and specificity before exploring

the potential clinical therapeutic or diagnostic use of such compounds as AC-178,335. Nevertheless, this antagonist may prove extremely useful in probing the role of SRIF in the physiological control of GH secretion, as well as its other biological effects.

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