Somatostatin Receptor Subtype Specificity in Human Fetal Pituitary Cultures

Differential Role of SSTR2 and SSTR5 for Growth Hormone, Thyroid-stimulating Hormone, and Prolactin Regulation

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Abstract

Somatostatin (SRIF), a hypothalamic inhibitor of pituitary growth hormone (GH) and thyroid-stimulating hormone (TSH) secretion, binds to five distinct receptor (SSTR) subtypes. We therefore tested SSTR subtype-specific SRIF analogs in primary human fetal pituitary cultures (23–25-wk gestation) to elucidate their role in regulating human pituitary function. Using reverse transcription-PCR, mRNA expression of SSTR2 and SSTR5 were detected in fetal pituitary by 25 wk. SRIF analog affinities were determined by membrane radioligand binding in cells stably expressing the human SSTR forms. GH secretion was suppressed equally (40–60%, $P < 0.005$) by analogs preferential for either SSTR2 (IC_{50} for receptor binding affinity, 0.19–0.42 nM) or SSTR5 (IC_{50}, 0.37 nM), and compounds with enhanced affinity for SSTR2 were more potent (EC_{50} for GH suppression, 0.05-0.09 nM) than Lanreotide® (EC_{50}, 2.30 nM) and SRIF (EC_{50}, 0.19 nM). Similarly, analogs with high affinity for SSTR2 or SSTR5 decreased TSH secretion (30–40%, $P < 0.005$). However, prolactin was effectively inhibited only by compounds preferentially bound to SSTR2 (20–30%, $P < 0.05$). Luteinizing hormone was modestly decreased (15–20%) by SSTR2- or SSTR5-specific analogs. An SSTR5-specific analog also exclusively inhibited GH in acromegalic tumor cells. Thus, SRIF regulation of GH and TSH in primary human fetal pituitary cells is mediated by both SSTR2 and SSTR5, both of which are abundantly expressed by 25 wk. In contrast, suppression of prolactin is mediated mainly by SSTR2. These results indicate that SSTR5 is critical for physiologic regulation of GH and TSH. SRIF analogs with selective affinity for this receptor may therefore be more effective in the treatment of hormone-secreting pituitary adenomas. (*J. Clin. Invest.* 1997. 99:789–798.) Key words: fetal pituitary • somatostatin receptor • growth hormone • thyroid-stimulating hormone • prolactin

Introduction

Somatostatin (SRIF),[1] a cyclic tetradecapeptide hormone originally isolated from mammalian hypothalamus, is a potent suppressor of pituitary growth hormone (GH) secretion (1). SRIF is synthesized as two bioactive peptides, SRIF-14 and SRIF-28, which are widely distributed throughout the central nervous system, endocrine, and peripheral tissues (2). The two peptides inhibit anterior pituitary hormone secretion (3), decrease cell proliferation (4), regulate endocrine secretions in the pancreas and gut (5), and modulate behavioral processes (6). Their short half life and the requirement for intravenous administration precluded their clinical use until the development of relatively stable long-acting analogs.

SRIF acts through a family of G-protein–coupled membrane receptors containing seven transmembrane domains. Five genes encoding distinct SRIF receptor (SSTR) subtypes have so far been cloned in human and other species (7–13) and termed SSTRs1–5 (14). The human SSTR subtype genes are located on different chromosomes (15), and SSTR1, 3, 4, and 5 lack introns, at least in their protein-coding sequence, while human SSTR2 generates two isoforms, a long (SSTR2A), and a short (SSTR2B) form through alternate mRNA splicing (16). The human SSTRs are 364–418 amino acid proteins with 42–60% identity among the five subtypes (15), with the highest amino acid sequence homology between human SSTR1 and SSTR4 (13). The receptors associate with different G-proteins and differ in their ability to mediate inhibition of adenylyl cyclase activity (9, 15, 17–19). Other postreceptor signal transduction systems, such as tyrosine phosphatase activation and ionic channel conductance are also associated with specific subtypes (15).

The different SSTR subtypes are widely expressed in rodent and human tissues. All five subtypes are expressed in the central nervous system (19, 20), and the hypothalamus contains all SSTRs. Receptors for SRIF have been localized on normal rat pituitary somatotropes, lactotropes, thyrotropes, gonadotropes, and corticotropes (21–23). Several studies in postmortem human pituitaries did not detect SSTR4 (24, 25) or SSTR3 (25), probably due to their low expression as compared with SSTR1, 2, and 5, while functional human pituitary adenomas, including GH-producing tumors, express SSTR1, 2, 3, and 5 (24–27).

The SSTRs 1–4 exhibit high and similar affinity for SRIF-

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1. *Abbreviations used in this paper:* GH, growth hormone; GHRH, growth hormone–releasing hormone; LH, luteinizing hormone; PRL, prolactin; RT, reverse transcription; SRIF, somatostatin; SSTR, somatostatin receptor; TSH, thyroid-stimulating hormone.

14 and -28, while SSTR-5 has greater affinity for SRIF-28 than for SRIF-14 (15). Recent pharmacological screening of a large panel of SRIF analogs has shown different binding affinities for each specific receptor subtype (28–30), and identified specific compounds with high affinities to SSTR2, SSTR3, or SSTR5. The SRIF analogs Octreotide® (SMS 201-995) and Lanreotide® (BIM-23014), which are used clinically to effectively control GH and thyroid-stimulating hormone (TSH) hypersecretion and pituitary tumor growth in patients with acromegaly or TSH-producing adenoma (4), bind with high affinity to SSTR2 and less efficiently to SSTR5.

GH synthesis and secretion by somatotrope cells is regulated by the hypothalamic hormones, GH-releasing hormone (GHRH), and SRIF. GHRH binds to specific receptors on somatotropes, increases intracellular cAMP, and selectively stimulates transcription of GH mRNA (31), and GH secretory pulses (32). SRIF suppresses basal GH secretion without altering GH mRNA levels (32). SSTR2 has been shown to mediate SRIF inhibition of GH secretion in rats (28), and SRIF appears to be the primary regulator of GH pulses in response to physiologic stimuli. Insulin-like growth factor-I, the peripheral target hormone of GH, participates in negative feedback regulation of GH by inhibiting both GH gene transcription (33) and GH secretion.

In this study, we used human fetal pituitary primary cultures to investigate regulation of anterior pituitary hormone secretion by SRIF analogs with different preferential binding affinities to the SSTRs, and also describe the pattern of SSTR subtype mRNA expression in these human pituitary cells. Correlation of specific analog affinity and suppression of GH secretion indicates selective inhibition of GH by SSTR5, in addition to SSTR2, in primary human pituitary cells. We also demonstrate the importance of SSTR5 in regulating GH in GH-secreting adenomas. The results also show the regulation by these analogs of TSH and prolactin (PRL) secretion, which are mediated by SSTR2, and in the case of TSH, also by SSTR5.

Methods

SRIF analogs. SRIF-14, BIM-23014 (Lanreotide®), BIM-23023, BIM-23052, BIM-23056, BIM-23268, BIM-23190, and BIM-23197 were provided by Biomeasure Inc. BIM-23268, an extended disulfide bridged ring-containing SRIF analog (Table I) designed to probe the effects of ring size on receptor preference, was synthesized by standard solid-phase methods on methylbenzhydrylamine resins, followed by hydrofluoric acid cleavage. BIM-23023, -23052, -23056, -23190, and -23197 were assembled by solid- or liquid-phase synthesis using conventional fluorenylmethyloxycarbonyl methods. BIM-23190 and -23197, based on earlier structures that demonstrated specificity for SSTR2 (Table I), were subsequently NH_2-terminally modified by solution-phase synthesis to increase the net positive charge of the molecule. The resultant peptide or peptide resin was treated with trifluoroacetic acid to liberate the linear peptides that were purified by reverse-phase HPLC. When appropriate, compounds were cyclized by treatment with iodine in methanol. Peptide content was determined by amino acid analysis. SMS 201-995 (Octreotide®) was obtained from Sandoz (East Hanover, NJ). Chemical and pharmacological characteristics of these compounds are shown in Table I. The analogs were dissolved in 0.01 M acetic acid and 0.1% BSA, and frozen at −20°C until used.

Stable expression of human SSTR subtypes. The complete coding sequences of genomic fragments of the human SSTR1–4 genes and a cDNA clone for human SSTR5 were subcloned into the mammalian expression vector pCMV. Clonal cell lines stably expressing hSSTRs1–5 were obtained by transfection into Chinese hamster ovaries (CHO)–K1 cells (American Type Culture Collection, Rockville, MD) using calcium phosphate coprecipitation. The plasmid pRSV-neo (American Type Culture Collection) was included as a selectable marker. Clonal cell lines were selected in RPMI media containing 0.5 mg/ml G418 (Gibco Laboratories, Grand Island, NY) and expanded into culture.

Radioligand receptor binding assays. Membranes from transfected CHO-K1 cells expressing the different hSSTR subtypes were obtained by cell homgenizing in ice cold 50 nM Tris-HCL buffer. For the hSSTRs 1, 3, 4, and 5 assays, aliquots of the membrane preparations were incubated (37°C/30 min) with 0.05 nM [^{125}I-Tyr^{11}]SRIF-14 in 50 mM Hepes (pH 7.4) containing BSA (10 mg/ml), $MgCl_2$ (5 mM), Trasylol (200 KIU/ml), bacitracin (0.02 mg/ml), and phenylmethylsuphonyl fluoride (0.02 mg/ml), in the presence of increasing amounts of unlabeled SRIF analogs. For the hSSTR2 assay, [^{125}I]MK-678 (0.05 nM) (28, 29) was employed as the radioligand (25°C/90 min). Incubations were terminated by rapid filtration through GF/C filters (presoaked in 0.3% polyethylenimine) using a Brandel filtration manifold. Each tube and filter was then washed three times with 5-ml aliquots of ice cold buffer. Specific binding was defined as the total radioligand bound minus that bound in the presence of 1,000 nM SRIF-14 (SSTRs 1, 3, 4, and 5), or 1,000 nM MK-678 for SSTR2.

IC_{50} values for the SRIF analogs studied (Table I) were calculated from the experimental data by nonlinear least-squares regression

Table I. Characteristics of SRIF Analogs

Compound	Molecular weight	Structure	SSTR binding affinity (IC_{50}-nM) 1	2	3	4	5	EC_{50} (nM) GH
SRIF-14	1638	Ala-Gly-c[Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys]	2.26	0.23	1.43	1.77	0.88	0.19
SMS 201-995	1019	D-Phe-c[Cys-Phe-D-Trp-Lys-Thr-Cys]-Thr-ol	1140	0.56	34.5	7030	7	
BIM-23014	1096	D-Nal-c[Cys-Tyr-D-Trp-Lys-Val-Cys]-Thr-NH_2	2330	0.75	107	2100	5.2	2.30
BIM-23023	1032	D-Phe-c[Cys-Tyr-D-Trp-Lys-Abu-Cys]-Thr-NH_2	7170	0.42	89	2700	4.18	0.09
BIM-23052	1122	D-Phe-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-NH_2	100	11.9	5.59	132	1.22	3.76
BIM-23056	1234	D-Phe-Phe-Tyr-D-Trp-Lys-Val-Phe-D-Nal-NH_2	337	132	177	234	12.1	5.13
BIM-23268	1078	c[Cys-Phe-Phe-D-Trp-Lys-Thr-Phe-Cys]-NH_2	18.4	15.1	61.6	16.3	0.37	0.06
BIM-23190	1202	N-hydroxyethylpiperazinyl-acetyl-D-Phe-c[Cys-Tyr-D-Trp-Lys-Abu-Cys]-Thr-NH_2	4577	0.34	217	> 1000	11.1	0.05
BIM-23197	1253	Hepes-D-Phe-c[Cys-Tyr-D-Trp-Lys-Abu-Cys]-Thr-NH_2	5547	0.19	26.8	> 1000	9.8	0.05

Abu, aminobutyric acid; Nal, β-(2-naphthyl)alanine.

analysis using the computer program RS1 (BBN Software Products Corp., Cambridge MA). Inhibition curves were fit to the four-parameter logistic equation described by DeLean et al. (34).

Human fetal pituitary and tumor tissue. Human fetal tissues of 23–25-wk gestation were obtained from an independent facility with third party referrals for therapeutic pregnancy termination, and with no direct or indirect involvement of our investigators in the termination referral. Studies of human fetal pituitaries followed guidelines of the National Advisory Board on Ethics in Reproduction (35). Written informed consent was obtained from pregnant subjects for anonymous distribution of aseptic tissue specimens. A specimen of an invasive GH-secreting pituitary adenoma was obtained at the time of transsphenoidal surgery.

Primary fetal pituitary and adenoma cell culture. Specimens (gestational age, 23–25 wk, both male and female) were harvested within 0.5–2 h of the termination procedure. Fetal pituitary was washed in low glucose DME supplemented with 0.3% BSA, 2 mM glutamine, and penicillin/streptomycin, and then minced and enzymatically dissociated using 0.35% collagenase and 0.1% hyaluronidase (both from Sigma Chemical Co., St. Louis, MO) for 45–60 min (36). Cell suspensions were filtered and resuspended in low glucose DME supplemented with 10% FBS, 2 mM glutamine, and antibiotics. For primary cultures, $\sim 5 \times 10^4$ cells were seeded in 48-well tissue culture plates (Costar Corp., Cambridge, MA) in 0.5-ml medium, and incubated for 72 h in a humidified atmosphere of 95% air/5% CO_2, at 37°C. Medium was then changed to serum free–defined low glucose DME containing 0.2% BSA, 120 nM transferrin, 100 nM hydrocortisone, 0.6 nM triiodothyronine, 5 U/liter insulin, 3 nM glucagon, 50 nM parathyroid hormone, 2 mM glutamine, 15 nM EGF, and antibiotics, and cells treated for 4 h with the SRIF analogs, after which, medium was collected and stored at −20°C for later hormone measurements. A single pituitary was divided and plated into 40–60 wells and used for a one-dose response experiment (0.01–100 nM) where five to six wells served as controls (treated with vehicle solution) and groups of four to five wells were treated with specific analogs. Thus for each concentration, all analogs were compared to each other. In addition, similar experiments were performed while pituitary cells were treated with 10 nM human GHRH (1-29)NH_2 (Biomeasure Inc.).

The GH-secreting tumor specimen was enzymatically dissociated and plated similarly. Adenoma cells were treated with 10 nM SRIF analogs (five to six wells for each compound) or with vehicle solution, in serum free–defined low glucose DME for 4 h.

Hormone assays. RIA for human GH and adrenocorticotropin hormone were performed in duplicate using kits (Diagnostic Products Corp., Los Angeles, CA), after 1:5 and 1:10 dilutions of the medium, respectively. Human TSH (after 1:2 dilution) and PRL (no dilution) levels were measured by immunoradiometric assay (Diagnostic Products Corp.). RIA for intact human luteinizing hormone (LH) (1:2 dilution) was performed using reagents provided by the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, MD).

Statistical analysis. Results are expressed as mean±SEM. Hormone suppression by each analog is expressed as a percentage of mean control basal concentration, in the same experiment, and differences were assessed by *t* test, where P values < 0.05 were considered significant. EC_{50} (half-maximal SRIF analog concentration for GH suppression) for each analog was calculated using an ALLFIT analysis. The high dose points at 100 nM were deleted from analysis because of apparent desensitization (the inhibition was partially reversed) at these high doses.

Somatostatin, GHRH, and IGF-I receptor mRNA expression. Human fetal pituitaries (18- and 25-wk gestation) were harvested and kept at −70°C for RNA extraction. After tissue homogenizing, total RNA was extracted using guanidinium isothiocyanate-phenolchloroform with TRizol (Gibco Laboratories) (37). RNA concentrations were estimated by ultraviolet spectrophotometry at 260 nm. Aliquots of RNA samples were electrophoresed through 1% agarose/1 × TBE gel stained with ethidium bromide to confirm RNA integrity. Reverse transcription (RT) followed by PCR amplification was performed to detect GH, SSTR2, SSTR5, GHRH receptor, and IGF-I receptor mRNA expression in the fetal pituitaries. 1 μg of each RNA sample was treated with 1 U deoxyribonuclease (DNase 1, amplification grade; Gibco Laboratories) for 15 min at 37°C, before the RT reaction to eliminate contaminating genomic DNA. The reaction was stopped by heating to 65°C for 10 min, and then cooled to room temperature and used in a 20-μl RT reaction containing 75 mmol/liter KCL, 50 mmol/liter Tris-HCL (pH 8.3), 3 mmol/liter $MgCl_2$, 10 mmol/liter DTT, 1 mmol/liter of each dNTP, 2.5 μmol/liter Oligo(dT)16, and 200 U reverse transcriptase (SuperScript II; Gibco Laboratories). The RT reactions were incubated at 42°C for 50 min, 70°C for 15 min, and 4°C for 5 min in the DNA Thermal Cycler 4800 (Perkin-Elmer Cetus Instruments, Emeryville, CA). Samples were also incubated without RT enzyme as negative controls. After the RT reaction, samples were treated with 2 U ribonuclease H (Gibco Laboratories) at 37°C for 20 min. Aliquots (2 μl) from the generated cDNA and the negative control reactions were used for subsequent PCR amplification of GH, SSTR2, SSTR5, GHRH receptor, and IGF-I receptor in reaction volumes of 100 μl containing 50 mmol/liter KCL, 20 mmol/liter Tris-HCL (pH 8.4), 1.5 mmol/liter $MgCl_2$, 200 μmol/liter of each dNTP, 40 pmol/liter of each primer, and 5 U Taq DNA polymerase (Gibco Laboratories). After initial denaturation step at 95°C for 5 min, amplifications were carried out for 40 cycles, with a final 7-min extension step at 72°C. Each cycle consisted of denaturation at 94°C for 1 min, annealing (1 min) at 60°C for GH, SSTR2, SSTR5, and GHRH receptor, and 58°C for IGF-I receptor, and elongation at 72°C for 75 s. The following primer sets were used: GH, 5′-CTTTTTGACAACGCTAGTCTC (exon 1, 453–473), and 3′-CAGGCTGTTGGCGAAGACACT (exon 3, 1013–1033) (38); SSTR2, 5′-GATGATCACCATGGCTGTG (480–498), and 3′-CAGGCATGATCCCTCTTC (1355–1372) (24); SSTR5, 5′-AACACGCTGGTCATCTACGTGGT (172–194), and 3′-AGACACTGGTGAACTGGTTGAC (361–382) (27); GHRH receptor, 5′-GGGTGAACTTTGGGCTTTTTCTCAA (932–956), and 3′-GCAGTAGAGGATGGCAACAATGAA (1159–1182) (39); IGF-I receptor, 5′-GCTGCTGGACCACAAACCGCT (exon 2, 656–676), and 3′-CCCTTGAAGATGGTGCATCCT (exon 4, 1098–1118) (40). The PCR products thus generated are 279, 893, 211, 251, and 463 bp for GH, SSTR2, SSTR5, GHRH receptor, and IGF-I receptor, respectively. PCR product of GHRH receptor was digested by Sal I (109- and 142-bp restriction products) and the IGF-I receptor by Bgl I (156 and 307 bp) (both enzymes from Gibco Laboratories), and was visualized with ethidium bromide after electrophoresis of 15 μl of the reaction solutions on 1.5% agarose gel.

Results

Receptor mRNA expression. RNA was extracted from human fetal pituitaries (18- and 25-wk gestation) and subjected to RT followed by PCR amplification. Results of the PCR reaction revealed the presence of both SSTR2 (893 bp) and SSTR5 (211 bp) PCR products (Fig. 1 *A*). Additionally, SSTR1 mRNA is present at this gestational age, but SSTR3 and SSTR4 are not expressed (data not shown). To confirm the pituitary origin of the mRNA, testing for GH expression in these tissues resulted in a 279 bp PCR product, as expected (Fig. 1 *A*). GHRH and IGF-I receptors, both mediating GH expression, are also well expressed at 25 wk (Fig. 1 *B*). The 251-bp band representing GHRH receptor expression was appropriately digested by Sal I (Fig. 1 *B*), and the IGF-I–receptor PCR product was cut by Bgl I to two shorter bands as expected (Fig. 1 *B*), thus confirming their specificity. All sample products were negative when reactions without RT were amplified by PCR. As SSTRs are intronless, this confirms that the positive

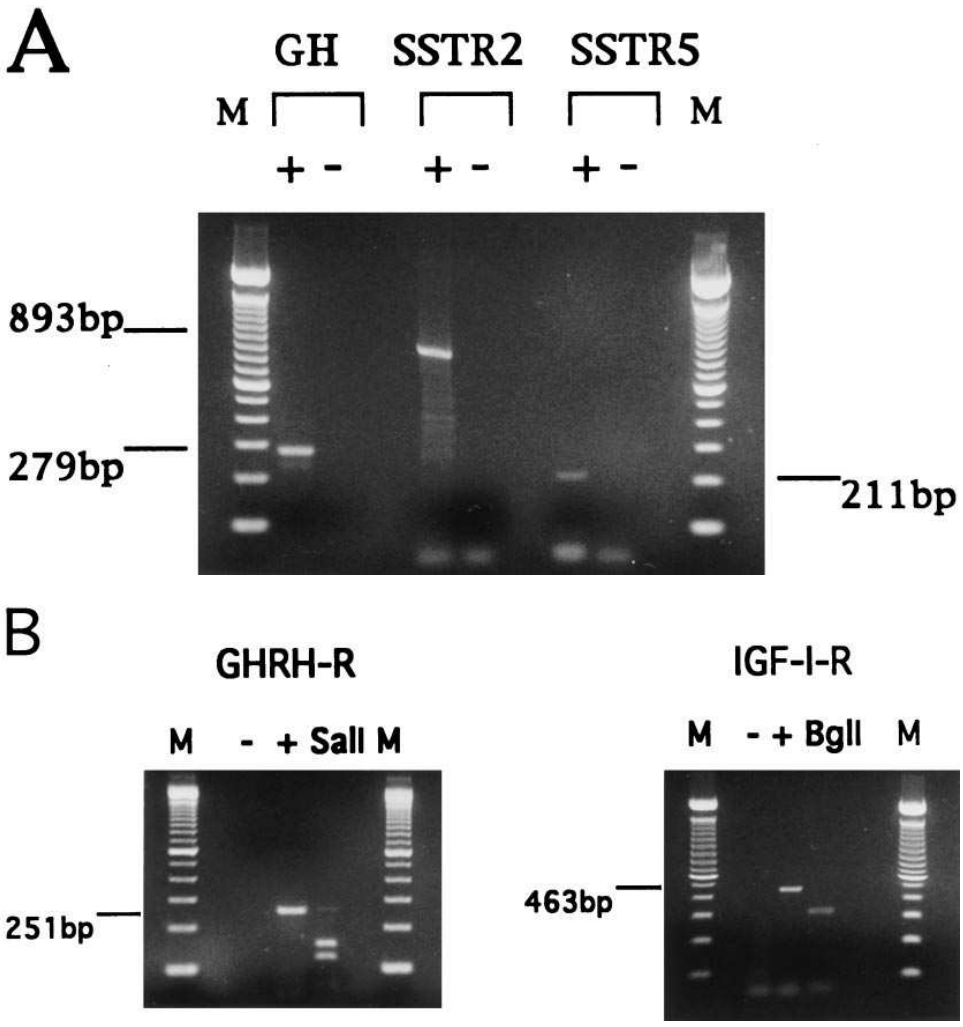

Figure 1. Somatostatin receptors (*A*), GHRH receptor (*GHRH-R*), and IGF-I receptor (*IGF-I-R*) mRNA expression (*B*) in 25-wk gestation human fetal pituitary. Extracted RNA (1 μg/reaction) was treated with deoxyribonuclease and subjected to reverse transcription using Oligo(dT) as primer. Samples incubated without RT enzyme served as controls. Aliquots from the generated cDNAs and the negative controls were subjected to subsequent PCR amplification (40 cycles) of GH, SSTR2, SSTR5, GHRH, and IGF-I receptors, using the primer pairs indicated in Methods. PCR products and enzyme digestion products were resolved on a 1.5% agarose gel. The expected PCR products of GH (279 bp), SSTR2 (893 bp), and SSTR5 (211 bp) are depicted in *A* (lane *M*, 100-bp DNA ladder; +, with RT; −, no RT added), and GHRH receptor (251-bp band, digested by Sal I), and IGF-I receptor (463-bp band, digested by Bgl I) are shown in *B*.

PCR product bands represent the presence of mRNA transcripts in the fetal pituitary.

Binding affinity of SRIF analogs for the human SSTRs. Affinities of the different SRIF analogs for the five human SSTRs are depicted in Table I. BIM-23023, -23190, and -23197 (cyclic octapeptides) have better affinity for SSTR2 (IC_{50}, 0.42, 0.34, and 0.19 nM, respectively) as compared with Octreotide® (IC_{50}, 0.56 nM) and Lanreotide® (IC_{50}, 0.75 nM), and BIM-23197 has comparable affinity to SSTR2 as the native SRIF-14 (IC_{50}, 0.23 nM). BIM-23052 (linear octapeptide) and BIM-23268 have low affinity for SSTR2 (IC_{50}, 11.9, and 15.1 nM, respectively), but relatively high affinity for SSTR5 (IC_{50}, 1.22, and 0.37 nM, respectively). Remarkably, BIM-23268 interacts with SSTR5 with better affinity than SRIF-14 (IC_{50}, 0.88 nM), and similarly to SRIF-28 (IC_{50}, 0.38 nM). Its selectivity for SSTR5 is 40-fold greater than that for SSTR2. BIM-23056 (linear octapeptide) has very low affinity for human SSTR2 and low affinity for SSTR5. Its interactions with SSTR1, SSTR3, and SSTR4 are also very poor.

Effects on GH. The analogs exhibited different inhibitory patterns of GH secretion by the human pituitary cells. BIM-23197, -23268, and -23190 were highly potent in suppressing GH, slightly better than SRIF-14, and significantly more potent than Lanreotide® (Figs. 2 and 3). BIM-23023 was almost as effective as BIM-23190. The potency of BIM-23052, however, was lower, but the analog still suppressed GH more effectively than did Lanreotide®, while BIM-23056 had a minimal effect on hormone regulation. Generally, the maximal inhibitory effect of the somatostatin analogs on GH release was achieved with concentrations of 10 nM (Fig. 2 *B*); BIM-23268 and -23197 suppressed GH secretion by 58% compared with untreated cells ($P < 0.0001$ for both analogs), BIM-23190 by 43% ($P < 0.005$), BIM-23023 by 48% ($P < 0.005$), while Lanreotide® had a modest effect (36%, $P < 0.01$) and SRIF-14 inhibited GH concentrations by 46% compared with untreated levels ($P < 0.01$). A similar pattern of suppression was observed when lower doses (0.1 and 1 nM) were employed (Figs. 2 *A* and 3). Lanreotide's® effect on hormone secretion was minimal, while BIM-23268 (33–36%, $P < 0.005$), BIM-23197 (32–40%, $P < 0.0005$), BIM-23190 (32–48%, $P < 0.005$), and BIM-23023 (29–45%, $P < 0.05$) were much more effective. Even SRIF-14 (23–41% suppression of GH, $P < 0.05$) was less potent than these latter compounds.

Interestingly, maximal GH suppression by the higher dose of analogs (10 nM) was more effective if the pituitary cultures were cotreated with GHRH (10 nM) (Fig. 4), achieving GH inhibition of 62% (BIM-23197), 60% (BIM-23190), 55% (both BIM-23268 and -23023), and 65% (SRIF-14), as compared

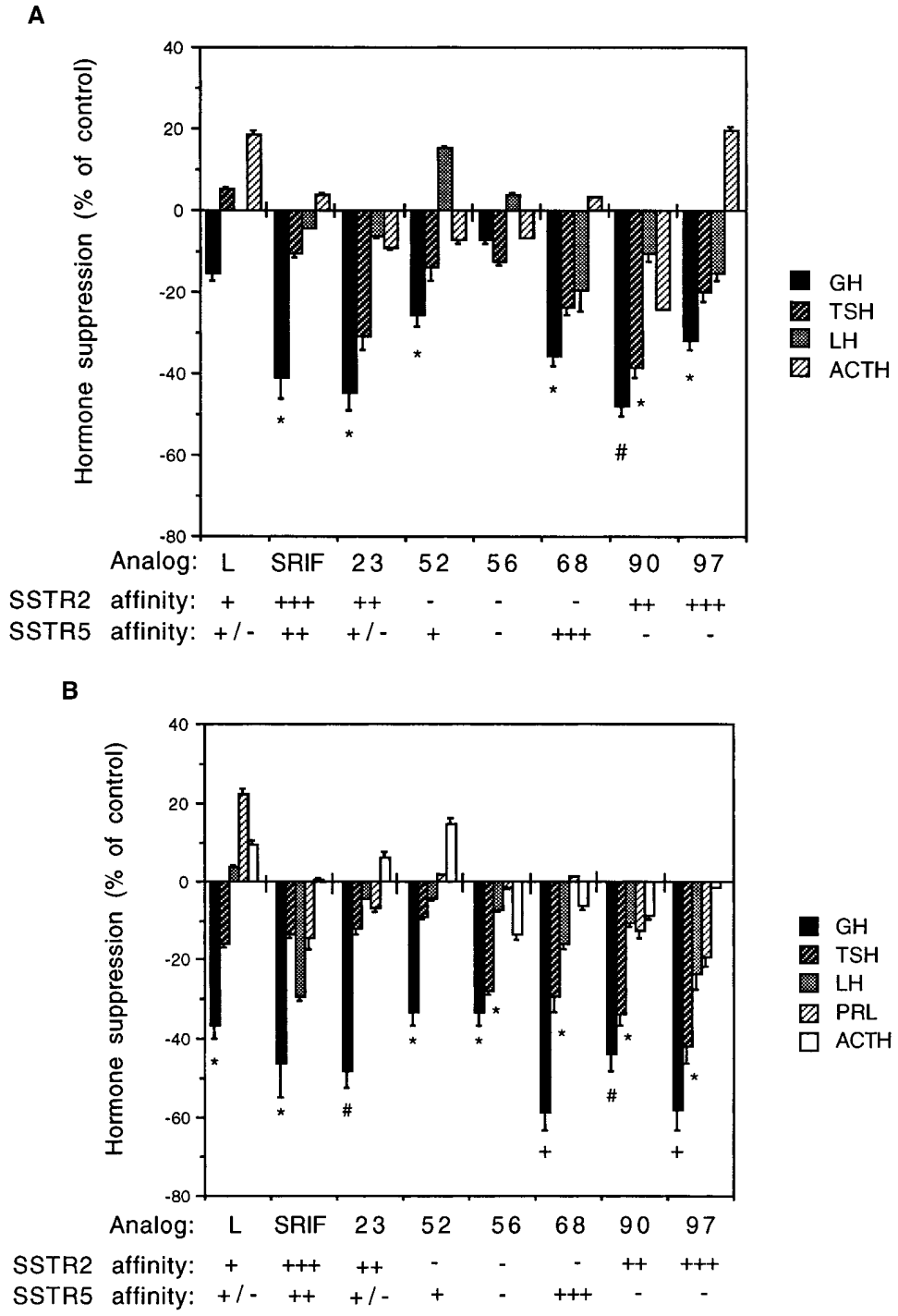

Figure 2. Human pituitary hormone suppression by SRIF analogs exhibiting different SSTR subtype specificity. Human fetal pituitaries were harvested between 23 and 25 wk gestation, cells were cultured (5×10^4 cells/well) and 72 h later treated with different SRIF analogs (1 nM, *A*; 10 nM, *B*) in serum-free defined medium for 4 h. Control wells were treated with vehicle solution. Each bar represents mean (±SEM) suppression of hormone secretion in four to five wells compared with control wells. L, Lanreotide®; SRIF, SRIF-14; 23, BIM-23023; 52, BIM-23052; 56, BIM-23056; 68, BIM-23268; 90, BIM-23190; 97, BIM-23197. $^*P < 0.05$; $^\#P < 0.005$; $^+P < 0.0001$.

with cells receiving GHRH alone ($P < 0.0001$ for all analogs). GHRH alone induced a twofold increase of GH secretion, as expected (41).

GH inhibitory curves of the different analogs are depicted in Fig. 3. All comparative suppression patterns were seen with 1 nM treatment. Maximal effect was usually achieved with 10 nM, and no further effect was seen with 100 nM peptide. The calculated EC_{50} of these compounds for GH suppression are shown in Table I. BIM-23190, -23197, -23268, -23023, and SRIF-14 had the lowest values (< 0.2 nM), while Lanreotide®, BIM-23052, and -23056 where significantly less potent (with midinhibitory concentrations 12–100-fold higher). To identify the SSTR subtype that might be associated with GH suppression in human somatotropes, we correlated analog potency to inhibit GH release with their respective binding affinities. As shown in Table I, analogs with very high affinity for SSTR2 (BIM-23190, -23197) were more potent (EC_{50} for GH suppression, 0.05 nM for both compounds) than those with mildly lower affinity (BIM-23023 and Lanreotide®). All these compounds did not differ in their affinities for SSTR1, 3, 4, and 5. However, BIM-23268 exhibiting low affinity for SSTR2, but with high binding to SSTR5, had a comparable effect on GH

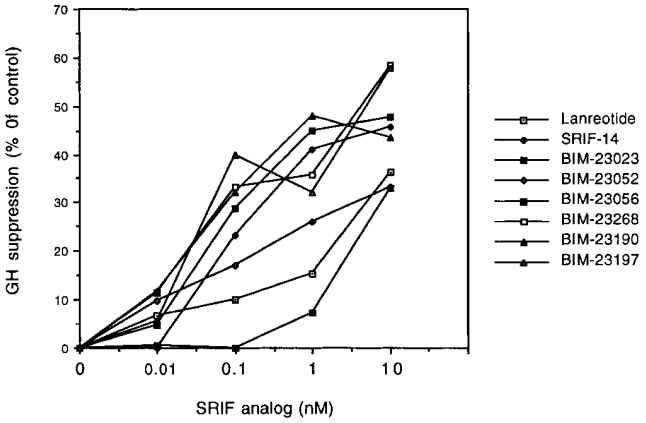

Figure 3. Human GH release from primary fetal pituitary cell cultures treated with SRIF analogs exhibiting different SSTR subtype affinities. For each indicated analog, a dose-dependent GH inhibition curve was plotted by combining the results of four experiments with the indicated doses. Maximal inhibition was achieved with 10 nM, and no further effect was seen with 100 nM. Each value represents mean suppression of GH secretion in four to five wells treated with the same analog compared with control wells in the same experiment.

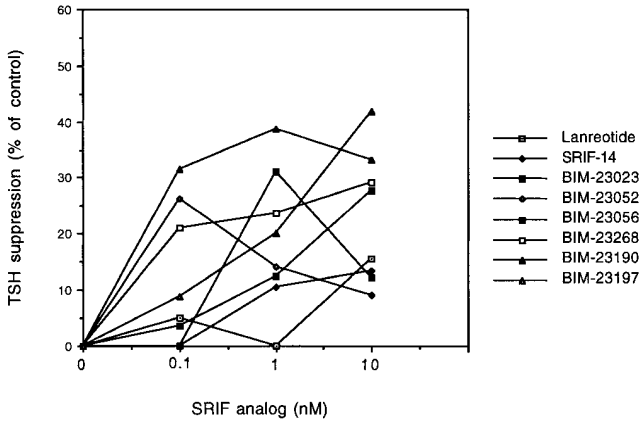

Figure 5. Dose-dependent human TSH suppression from primary fetal pituitary cultures treated with SRIF analog compounds. Each curve is a combination of three doses of the indicated compound. Each value represents mean inhibition of TSH release in four to five wells compared with control cells from the same fetal pituitary.

release (EC_{50}, 0.06 nM), as did the analogs with the highest affinity for SSTR2. In addition, BIM-23052 with low affinity for SSTR2, but higher for SSTR5, usually suppressed GH as effectively as did Lanreotide®. Thus, these results indicate that both SSTR2 and SSTR5 are involved in the regulation of GH release from the human anterior pituitary.

Effects on TSH. SRIF and its analogs suppressed TSH secretion significantly but less potently as compared with GH suppression (Figs. 2 and 4). Generally, the potent compounds were BIM-23190 (40% suppression, $P < 0.005$), BIM-23197 (32%, $P < 0.01$), BIM-23268 (29%, $P < 0.005$), and BIM-23023 (28%, $P < 0.05$) (mean TSH suppression in four different experiments), compared with Lanreotide® (12%, NS), SRIF-14 (25%, $P = 0.06$), and BIM-23052 (20%, $P = 0.05$) (Fig. 5). Thus, SSTR5, in addition to SSTR2, mediates regulation of TSH secretion.

Effects on PRL. In vitro PRL secretion from human fetal lactotropes is suppressed by SRIF-14 and its analogs (Figs. 2 and 4). Compared with GH and TSH suppression, the inhibitory effect on PRL is mild, but significant, and achieved at a dose of 10 nM. Remarkably, the analogs with higher potency for altering PRL secretion are those with better affinity for SSTR2: BIM-23190 (29% suppression), BIM-23197 (28%), SRIF-14 (23%), and BIM-23023 (20%) (mean PRL suppression in three different experiments, $P < 0.05$ for these ana-

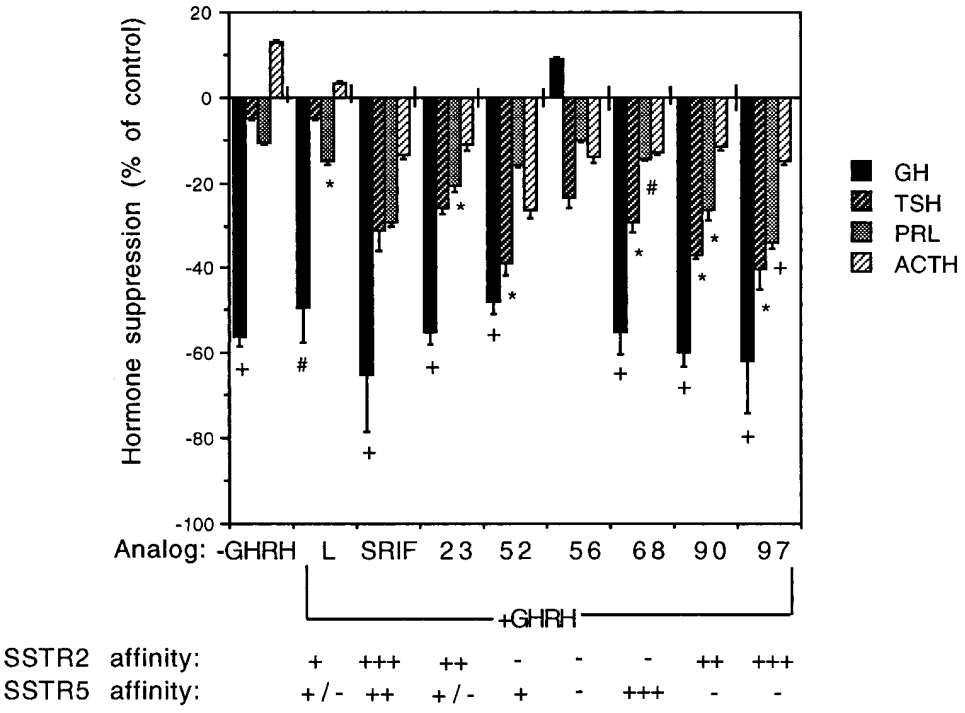

Figure 4. Human fetal pituitary hormone suppression in primary cell cultures cotreated with different SRIF analogs (10 nM) and human GHRH (1-29)NH_2 (10 nM) in serum free defined medium for 4 h. Control wells were treated with GHRH only. Each bar represents mean±SEM suppression of hormone secretion in four to five wells compared with control wells. -GHRH represents cells incubated without SRIF analogs or GHRH (vehicle only). L, Lanreotide®; SRIF, SRIF-14; 23, BIM-23023; 52, BIM-23052; 56, BIM-23056; 68, BIM-23268; 90, BIM-23190; 97, BIM-23197. $^{*}P < 0.05$; $^{\#}P < 0.005$; $^{+}P < 0.0001$.

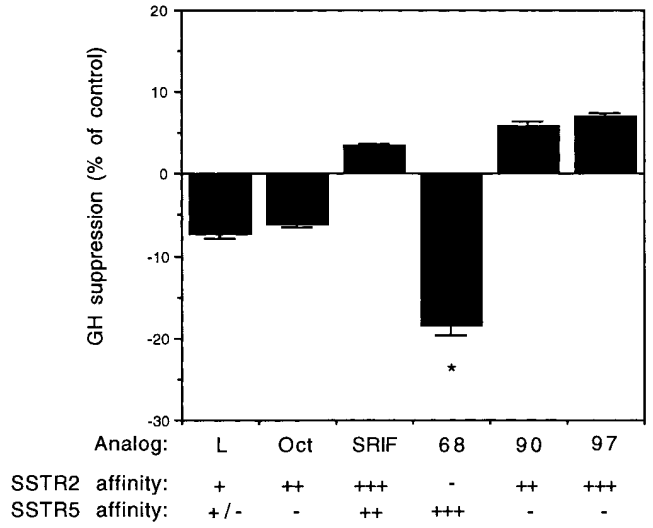

Figure 6. Human GH suppression in primary cultures derived from an invasive GH-secreting adenoma treated with SRIF analogs (10 nM) for 4 h in serum-free defined medium. L, Lanreotide®; Oct, Octreotide®; SRIF, SRIF-14; 68, BIM-23268; 90, BIM-23190; 97, BIM-23197. *$P < 0.05$.

logs). However, Lanreotide® (with lower affinity to SSTR2) was ineffective, as were BIM-23268 and BIM-23052, which have low affinity for SSTR2 but better affinity for SSTR5. Thus, SSTR5 is not involved in PRL regulation in primary human fetal lactotropes, and SSTR2 is the only receptor subtype to mediate the inhibitory effect of the potent analogs on PRL.

Effects on LH and ACTH. LH concentrations in the medium were modestly effected by some analogs (Fig. 2). Lanreotide® did not alter LH secretion, but BIM-23268, -23190, and -23197 demonstrated 15–20% suppression of untreated levels. Other compounds had no effect on LH. ACTH was not suppressed by SRIF and its analogs (Fig. 2), but very mild inhibition (up to 15%) was induced by most compounds when fetal cells were also cotreated with GHRH (10 nM) (Fig. 4).

Tumor GH suppression. Treatment of dispersed cells derived from an invasive GH-secreting adenoma resulted in GH suppression only in those cells exposed to BIM-23268 (20%, $P < 0.01$) (Fig. 6), while other analogs had no significant effect on GH secretion in this aggressive tumor. Thus, these results emphasize the important contribution of SSTR5 to GH regulation in pituitary tumor cells, as this specific adenoma was resistant to analogs selective for SSTR2, and responded only to a compound with enhanced affinity for SSTR5.

Discussion

This study shows that SRIF suppression of GH and TSH secretion in primary human pituitary cultures is mediated through ligand binding to two SRIF receptor subtypes, SSTR2 and SSTR5. However, PRL secretion in fetal human lactotropes is reduced via selective binding to SSTR2 only. Novel SRIF analog compounds with improved affinity for SSTR2 or SSTR5 suppressed GH, TSH, PRL, and LH more efficiently compared with the current clinically used analog Lanreotide®, and even slightly better than the naturally occurring peptide SRIF-14. In addition, treatment of primary cultures of an invasive GH-secreting adenoma resulted in GH suppression only with a compound selective for SSTR5. Therefore, new analogs with enhanced affinity to SSTR2 and/or SSTR5 may improve control of hormonal oversecretion (GH, TSH, and PRL) when the currently available analogs are not efficacious.

For our studies, we used primary cultures of human fetal pituitaries, at 23–25 wk of gestation. At this age, all hormone-producing cells including corticotropes, somatotropes, gonadotropes, and thyrotropes are well differentiated (42–43). Lactotropes, however, are only identified after a 24-wk gestation. Until that time, PRL is produced solely by mammosomatotropes, bihormonal primitive stem cells that secrete both PRL (not before 14 wk) and GH (42). Corticotropes are the first hormone-secreting cells to be recognized in the anterior pituitary, by 6 wk of gestation, and GH immunoreactivity is identified in human well differentiated somatotropes by 8 wk (42). Thyrotropes and gonadotropes expressing TSH and gonadotropins appear in the human adenohypophysis between 12 and 15 wk (42, 43). The hypothalamic–hypophysial portal vascular system is well established by 18–20 wk (44), and immunoreactive somatostatin is present with increasing concentrations in fetal hypothalamus by 12–26 wk (45). Human pituitary cells are responsive to hypothalamic releasing and inhibitory factors, including SRIF (43, 46), GHRH (43, 46), and other hypothalamic hormones (43) as early as 10–14 wk, with significant increase in the magnitude of somatotrope response to SRIF and GHRH with increasing fetal age. IGF-I also decreased GH release in human fetal pituitaries aged 11–20 wk (47). Thus, responsiveness of fetal somatotropes to hormonal regulation indicates functional maturity of the hypothalamic–hypophysial axis early in gestation.

It was previously shown by RT-PCR in a single fetal pituitary (14 wk) that all five human SSTR subtype mRNAs were expressed (24). We here demonstrate the exclusive expression of SSTR2 and 5, the SRIF receptors involved in pituitary hormone suppression, at the age of 18–25 wk (Fig. 1 *A*), and also GHRH and IGF-I receptor mRNA in human fetal pituitary tissue (Fig. 1 *B*). We used RT-PCR for mRNA expression studies, as Northern or ribonuclease protection assays are limited by the quantity of RNA required for analysis. Thus, to study pituitary hormone regulation by SRIF analogs, we used fetal pituitary cell cultures as a unique model of functional human pituitary tissue, which already contains mature hormone-producing cells, expresses receptors for releasing and inhibitory factors, and responds appropriately to these regulating hormones.

Circulating GH suppression after Octreotide® administration to patients with GH-secreting tumors closely correlates with the density of SSTRs on excised tumor tissue (48), and with the presence of receptors as demonstrated in vivo by radiolabeled Octreotide® scan (49). Octreotide® and Lanreotide® have high affinity for SSTR2, and lower affinity for SSTR5 (Table I). Pituitary GH- and TSH-producing tumor responsiveness to the analogs is explained by the common presence of these receptor subtypes in the tumors (24–27), but there has been no indication of which receptor is directly involved in pituitary hormone regulation, either in normal human or in pituitary tumor cells. Moreover, SSTR1 has been detected in the human (24, 25) and rat (22) putuitary, as well as pituitary adenomas (24–26), but its involvement in hormone regulation is unclear. In the rat, SSTR2 was shown to be involved in GH

suppression, using a panel of somatostatin analogs with differential affinities for different SSTRs (28). GH suppression by the compounds was highly correlated with their affinity for SSTR2. However, SSTR4 and 5 were not then studied, although rat somatotropes predominantly express these receptor subtypes, which are more abundant than SSTR2 mRNA (22, 23). Moreover, species-specific differences in SSTRs mRNA expression and function do exist, and in fact human SSTR5 has a 160-fold lower affinity for Octreotide® than the rat receptor (50). Thus, these studies in rodents are not directly relevant to the human pituitary and we therefore studied the role of the different SSTRs in human hormone regulation using primary human pituitary cells.

We now show for the first time that both SSTR2 and SSTR5 individually contribute to the function of SRIF to inhibit GH release. Using SRIF analogs with either relative selectivity for SSTR2 or SSTR5, we demonstrate the effects of ligand binding to each receptor subtype on GH suppression. We used a group of analogs (BIM-23190, -23197, -23023) with significantly higher affinity for SSTR2, which suppressed human GH more efficiently than the clinically used SRIF analog drug Lanreotide®, and even slightly better than SRIF-14, one of the biologically active members of the SRIF family. BIM-23190 and -23197 are two- to fourfold more selective for SSTR2 than currently available SSTR2 agonists, such as Lanreotide® and Octreotide®, and are also more potent in inhibiting GHRH-induced GH release in the rat (51). In addition, these compounds are stable to rat in vivo degradation, with no binding to plasma proteins (51). We also tested analogs with improved affinity for SSTR5 (BIM-23268, a compound discovered to have the highest affinity and greatest specificity observed so far for human SSTR5, and BIM-23052) (52), which decreased GH release from fetal somatotropes to the same degree as did the SSTR2 analogs. BIM-23268 structure differs from that of the other cyclic octapeptide analogs in that the disulfide bridge begins and ends at the NH_2 and COOH terminals of the peptide, rather than positions 2 and 7 (Table I). Stimulation with GHRH at physiologic concentrations improved the ability of the SRIF analogs to achieve more potent inhibition of GH. The association between the potencies of these compounds to inhibit in vitro release of human GH and the abilities of these analogs to inhibit radioligand binding to SSTR2 or SSTR5 indicates that both these receptors have a role in mediating GH suppression by SRIF. This implies that, using this new generation of analogs with improved affinity for SSTR2 or SSTR5 in patients with acromegaly, improved suppression of GH may be achieved. The GH-secreting pituitary tumor cells that responded exclusively to BIM-23268 in vitro (Fig. 6) highlight the advantage that analogs with enhanced affinity for SSTR2 or SSTR5 may have in treating resistant pituitary adenomas. Further data on GH suppression by these analogs in primary cultures of GH-producing adenomas are required.

Compared with effects on GH secretion, the magnitude of TSH suppression was about two-thirds that of GH inhibition, and as for GH, compounds with higher affinity for SSTR2 or SSTR5 had relatively better effects on TSH secretion. Thus, the results indicate that these two receptors also contribute to TSH regulation. Effects of SRIF on in vitro TSH release from bovine pituitary (53), and in vivo in normal volunteers (54), are well established, as are the in vitro and in vivo effects on TSH levels in TSH-secreting pituitary adenomas (55). In healthy subjects, SRIF and Octreotide® also have a greater in vivo inhibitory effect on GH as compared to TSH secretion (56). However, data on specific SSTR's involvement or analogs with improved suppression of human TSH have not been reported. These results illustrate the potential for use of analogs with improved affinity for SSTR2 or SSTR5 as a promising medical modality for treatment of TSH-secreting tumors, which usually are not effectively managed by surgery or radiotherapy alone.

In contrast to GH and TSH suppression, which is mediated by SRIF analogs via both SSTR2 and SSTR5, PRL release from human fetal lactotropes or mammosomatotropes is affected mainly through ligand binding to SSTR2. PRL suppression was mild but significant compared with that achieved for GH and TSH and the new compounds with better affinity for SSTR2 inhibited PRL with greater potency than did SRIF-14 or Lanreotide®. In neonatal (57) and adult rat pituitary cell cultures, SRIF does not inhibit PRL synthesis or release (58), but effectively suppresses PRL synthesis only after estrogen priming (58). PRL release from primary cultures of human fetal pituitaries responds to GHRH stimulation and to SRIF suppression in parallel with GH (42), consistent with production of both hormones by mammosomatotrope cells. In normal humans, no significant decrease of either basal or TRH-stimulated PRL levels occurs in vivo during SRIF infusions (56). Rat lactotropes express both SSRT2 and 5 (22, 23), but data regarding normal human lactotropes are lacking. We used fetal pituitaries that contain mammosomatotropes, primitive cells that may express different profiles of SSTR subtypes than do adult lactotropes, and thus respond to the potent SRIF analogs with PRL suppression. Alternatively, the improved effect of these analogs on human lactotropes results from their pharmacological characteristics; i.e., improved binding to the pivotal receptor SSTR2. Thus, these new analogs may be tested in patients with prolactinomas, which normally do not respond to SRIF analogs, to suppress PRL secretion from adenomatous tissue. Understanding the differential effects on hormone regulation mediated via different SSTRs is important in designing compounds with different receptor affinities for treatment of distinct pituitary pathologies.

LH secretion from fetal pituitary cells was mildly suppressed by the potent analogs with high affinity for SSTR2 and SSTR5. In both male and female healthy subjects, SRIF decreased LH pulse amplitude by 30–35%, with no significant change in pulse frequency (54). Moreover, nonfunctioning pituitary adenomas contain SSTR2 and 5 (24–27), and SRIF treatment of primary cultures from nonfunctioning tumors suppressed both intact gonadotropin and β-subunit secretion in 30–60% of hormone-producing tumors (59). In addition, SRIF infusion to patients with α-subunit–secreting tumors resulted in a significant decrease in serum α-subunit levels in most patients (60). As potent SRIF analogs are shown here to suppress LH secretion in normal pituitary cells, these compounds may have a role in suppressing glycoprotein hormone and subunit secretion in clinically nonfunctioning pituitary adenomas.

Our results illustrate the important role of SSTR2 and SSTR5 in mediating human anterior pituitary hormone suppression by SRIF. Interestingly, normal human pancreatic islets express SSTR1, 2, and 4, but not SSTR5 (61), and normal pancreatic β cell secretion is mediated via SSTR2, but not SSTR5 (62). In addition, human endocrine tumors affected by

SRIF, including insulinomas, glucagonomas, carcinoids, and pheochromocytomas also do not express SSTR5 (61). Thus, SSTR5 involvement in hormone regulation is probably a unique characteristic of the pituitary and other endocrine tissues, including the pancreatic islets and the adrenal gland, which normally do not express SSTR5 (61), mediate SRIF effects on hormone secretion via SSTR2 only.

SSTR5 contribution to the physiologic negative feedback control played by SRIF on pituitary hormone secretion is significantly important, as a new generation of potent SRIF analogs with high affinity for SSTR5 may be used to improve control of hormone hypersecretion in patients with GH-, TSH- and even PRL-producing adenomas. However, as the SSTR subtype profile and postreceptor signal pathways of pituitary tumors may differ from normal pituitary, and tumors that secrete the same hormone may express different receptor subtypes, these analogs should be tested both in vitro to suppress hormone secretion from primary cultures of hormone-secreting adenomas and in vivo in patients harboring these tumors.

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