Somatostatin Receptor Type 5 Modulates Somatostatin Receptor Type 2 Regulation of Adrenocorticotropin Secretion*

Somatostatin inhibits adrenocorticotropin (ACTH) secretion from pituitary tumor cells. To assess the contribution of somatostatin receptor subtype 5 (SST5) to somatostatin receptor subtype 2 (SST2) action in these cells, we assessed multipathway responses to novel highly monoreceptor-selective peptide agonists and multireceptor agonists, including octreotide and somatostatin-28. Octreotide and somatostatin-28 cell membrane binding affinities correlated with their respective SST2-selective peptide ligand. Although octreotide had similar inhibiting potency (picomolar) for cAMP accumulation and ACTH secretion as an SST2-selective agonist, somatostatin-28 exhibited a higher potency (femtomolar). Baseline spontaneous calcium oscillations assessed by fluorescent confocal microscopy revealed two distinct effects: SST2 activation reduced oscillations at femtomolar concentrations reflected by high inhibiting potency of averaged normalized oscillation amplitude, whereas SST5 activation induces brief oscillation pauses and increased oscillation amplitude. Octreotide exhibits an integrated effect of both receptors; however, somatostatin-28 exhibited a complex response with two separate inhibitory potencies. SST2 internalization was visualized with SST2-selective agonist at lower concentrations than for octreotide or somatostatin-28, whereas SST5 did not internalize. Using monoreceptor-selective peptide agonists, the results indicate that, in AtT-20 cells, SST5 regulates the dominant SST2 action, attenuating SST2 effects on intracellular calcium oscillation and internalization. This may explain superior somatostatin-28 potency and provides a rationale for somatostatin ligand design to treat ACTH-secreting pituitary tumours.

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Anat Ben-Shlomo‡, Kolja A. Wawrowsky‡, Irina Proekt‡, Nathaniel M. Wolkenfeld‡, Song-Guang Ren‡, John Taylor§, Michael D. Cullers‡, and Shlomo Melmed‡‡

From the ‡Department of Medicine, Cedars Sinai Research Institute, David Geffen School of Medicine, UCLA, Los Angeles, California 90048 and §Biomeasure Inc., Milford, Massachusetts 01757

Somatostatin (somatotropin-release inhibiting factor, SRIF),1 suppresses hormone release from secreting cells, including pituitary corticotrophs (1, 2). Selective SST receptor subtype ligands regulate pituitary hormones in vitro (3–5). Deletion of mouse somatostatin receptor type 2 (SST2) (6) resulted in increased pituitary ACTH release (7) underscoring the physiologic role for SRIF in regulating ACTH (8, 9). Mouse pituitary AtT-20 corticotroph cells are the only viable cell line available to study ACTH secretion in vitro. These cells express endogenous somatostatin receptors (SSTs) and respond to SRIF with a dose-dependent inhibition of ACTH secretion. AtT-20 cells express specific binding sites for SST2 and SST5, but not for SST1, -3, or -4 (10, 11). Through these receptors, SRIF inhibits pituitary cell adenylate cyclase activity and blocks inward Ca2+ currents (12–14). Unfortunately, the currently available SRIF agonist, octreotide, which recognizes primarily SST2, is ineffective in treating ACTH-secreting pituitary adenomas (15). Therefore, elucidating pathways whereby SRIF activates corticotroph receptors will facilitate design of effective SRIF analog ligand-selective pharmacotherapy (1) for the inexorable ACTH hypersecreting-characterizing patients with Cushing disease.

Baseline spontaneous Ca2+ oscillations (BSCOs) occur in resting pituitary cells, including gonadotroph, somatotroph, and corticotroph cells (16–24). Only minor differences in baseline and stimulated calcium oscillation patterns were noted between normal corticotroph and AtT-20 cells (16). Mechanisms underlying baseline intracellular calcium oscillations and their role in resting corticotroph cell biology are not clear. BSCOs may represent the sum of cellular calcium channels mediating replenishment and maintenance of calcium concentrations required for intact calcium-dependent signaling pathways and cellular homeostasis (25). Their importance for regulation of ACTH secretion has also been suggested (16, 21, 26). SRIF inhibits calcium currents in AtT-20 cells by several mechanisms, including increased inward K+ flux through rectifying K+ channels and cell membrane hyperpolarization (22), blocking of Ca2+ influx through L-type Ca2+ channels (23, 27), and possibly baseline cAMP oscillations (22, 28). To date, published studies have not employed selective SST2 or SST5 peptide agonists to study the individual contribution of each of these receptor subtypes to corticotroph cell SRIF responses, but have utilized peptide agonists with affinity to both SST2 and SST5 (27), all SSTs (22, 29, 30), or non-peptide agonists selective for SST2 but not to SST5 (10, 11). Human and mouse SST5 rapidly internalize upon SRIF-14 treatment (31–36); whether or not SST5 is internalized, remains unclear (31, 33, 37, 38).

In this study we assessed individual and combined contributions of SST2 and SST5 to SRIF signaling in AtT-20 cells and compared different ligand-mediated pathways, namely ligand binding, ACTH secretion, cAMP accumulation, spontaneous calcium oscillations, and receptor internalization. The results show that selective SST5 activation regulates SST2 signaling,
implying a requirement for SST5 agonism in treating patients with ACTH-secreting adenomas.

**MATERIALS AND METHODS**

**Ligands**—Peptide compounds were obtained from BioMearce Inc. (Milford, MA). BIM-23120 and BIM-23206 are SST2- and SST5-selective peptide agonists, respectively. BIM-23454 is an SST2 antagonist (39). Stock solutions (1 mM) of compounds were prepared in 0.01 M acetic acid, stored at −80 °C, and diluted with DMSO. Orotic acid-SRF-28 (Phoenix Pharmaceuticals Inc., Belmont, CA) were diluted in sterile H2O as 100 μM stock. SRIF-28 and SRIF-14 purity is 98.5%, with a verified disulfide bond. All ligands were stored at −20 °C until used within 6 months, and were thawed once.

**Plasmid cDNA**—Human SST2 (hSST2) and SST5 cDNAs, kindly provided by Dr. G. I. Bell (Howard Hughes Medical Institute, University of Chicago). hSST2 was amplified by PCR from a hSST2-pGEM3Z plasmid using Roche High Fidelity Enzyme systems (Roche Applied Science). Sense and antisense primers were designed to introduce HindIII and Sall restriction sites, respectively, in-frame with the hSST2 coding sequence. Primers were: sense, 5′-AAG CTT GCC ATG AGC ATG GAT CAG-3′; antisense, 5′-GTG CAC GCC GAT CAG GGC CTT TGC CTG-3′. PCR products were subcloned into pcRII-TOPO TA cloning vector (Invitrogen). The HindIII-Sall insert was subsequently excised by double digest and subcloned into the peYFP-N1 or peCFP-N1 vectors (Clontech, Palo Alto, CA), to create an hSST2 construct expressing eYFP or eCFP fluorophores fused to its C terminus. The hSST2-eYFP-N1 and hSST2-eCFP-N1 fusion constructs were verified by sequencing. hSST5 was amplified by PCR from a hSST5-pGEM3Z plasmid using Roche High Fidelity Enzyme systems. Sense and antisense primers were designed to introduce HindIII and Sall restriction sites, respectively, in-frame with the hSST5 coding sequence. Primers were: sense, 5′-AAG CTT GCC ATG AGC TTG CTG GTC TGC-3′; antisense, 5′-GTC GAC GCC AGC TTG CTG GTC TGC-3′. The HindIII-Sall insert was subsequently excised by double digest and subcloned into the peYFP-N1 vector (Clontech), to create an hSST5 construct expressing eYFP fluorophore fused to its C terminus. The hSST5-eYFP-N1 fusion construct was verified by sequencing. Commercially available and sequenced 3XHA-tagged hSST5 (UMR cDNA resource center, www.cdna.com) was then cloned into the HindIII restriction site of the peYFP-N1 vector (Clontech). hSST5-eYFP fusion protein was expressed in 293T cells with an intermediate re-suspension in calcium-free buffer. Final pellets were re-suspended in 10 mM Tris-HCl for assay. Aliquots of membrane preparations were incubated (90 min at 25 °C with 0.05 mM [125I]SRIF-14 in 50 mM HEPES (pH 7.4) containing BSA (0.2%); MgCl2 (5 mM) in 0.3 M. Internal standards were incubated with rapid filtration GF filters (pre-soaked in 0.3% polyethylimine) using a Brandel filtration manifold. Each tube and filter was washed three times with 5-ml aliquots of ice-cold buffer. Specific binding was defined as total radioactivity bound minus that bound in the presence of unlabeled ligand.

**Hormone Assays**—For ACTH and cAMP measurements cells were co-treated with forskolin (20 μM, Sigma-Aldrich) and the ligand of interest for 1 h in low glucose Dulbecco’s modified Eagle’s medium supplemented with 0.3% BSA and 1% antibiotic/antimycotic. Secreted ACTH concentrations in culture medium were measured by radiimmunomassay with a measurable range of 10–100 pg/ml (ICN Pharmaceuticals, Inc. Costa Mesa, CA). cAMP was extracted (40) and measured by radiolimmunoassay (measurable range 2.5–100 fmol) using cAMP, 2-O-[[125I]iodotyrosine methyl ester (PerkinElmer Life Sciences), and rabbit anti-cAMP antibody (Fitzgerald, Concord, MA).

**Calcium Analysis by Confocal Microscopy**—Half an hour before visualization, cells were suspended in 1 μM Calcium Green-1 AM 488 dye (Molecular Probes, Eugene, OR) (41) dissolved in anhydrous dimethyl sulfoxide and nonionic detergent Pluronic F-127 (10% solution in water) (41), then washed and placed in the confocal microscope incubator chamber. Cells were imaged with a Leica TCS SP confocal microscope (Leica Microsystems, Heidelberg, Germany), and samples were excited with the 488 nm line of the argon laser. Scanning rate was set to 3 frames per second for 280 s (840 frames) per time course or single ligand dose. For dose response measurements, cells were screened for 45–50 min during which a 10-fold higher dose of the ligand was added to the cells every 5 min. To minimize bleaching samples were imaged with 1% ethanol (pH 7.5, 0.05 μM) units, wide spectral detection window, and Acusto-optical-tunable-filter set to 9%. Images represent single optical slices, and intensity graphs for regions of interest were generated with confocal analysis software. Calcium oscillations for each concentration were analyzed using ImageJ software (www.rsb.info.nih.gov), and the average percent change (42, 43) from the median intensity (defined as averaged normalized oscillations amplitude,) was plotted to a response curve. For each cell analyzed: 86 cells for the untreated group, 43 cells for nimodipine, 84 cells for SST2-selective agonist, 52 cells for SST5-selective agonist, 64 cells for octreotide, and 58 cells for SRIF-28 treatment. Baseline spontaneous Ca2+ oscillations (BSCOs) pauses are defined as periods of very low oscillation amplitudes empirically defined as <2%. Randomly defined pauses as brief or short (<100 frames or 30 s) and long (>300 frames or 31 s).

**Immunoprecipitation and Western Blot Analysis**—AtT-20 cells were grown to confluence in 6-well plates, washed with phosphate-buffered saline (pH 7.0), and lysed with 500 μl of non-detergent lysis buffer (10 mM Tris-HCl, 5 mM EDTA, 3 mM EGTA, 250 mM sucrose, pH 7.6) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 2 μg/ml aprotinin) and 1 mM sodium pyrophosphate, 10 mM sodium orthovanadate, pH 7.5 containing protease inhibitors and 1% Triton X-100 and lysed for 1 h on ice with occasional agitation. The lysate was spun for 30 min at maximum speed to remove insoluble material, and the supernatant was used for immunoprecipitation. 500 μl of membranal fraction lysate was pre-cleared with 50 μl of Sepharose-protein A beads (Sigma-Aldrich). hSST2-eYFP fusion protein was immunoprecipitated by incubating solubilized fractions overnight at 4 °C with a monoclonal anti-GFP antibody (Roche Applied Science) pre-bound to Sepharose-A beads. Immune complexes were collected by centrifugation, washed three times with buffer containing 30 mM Heps, 30 mM NaCl, 10 mM EDTA, 10 mM sodium pyrophosphate, 10 mM NaF, 2 mM sodium orthovandate, pH 7.5 containing protease inhibitors and 1% Triton X-100 and lysed for 1 h on ice with occasional agitation. The lysate was spun for 30 min at maximum speed to remove insoluble material, and the supernatant was used for immunoprecipitation. 500 μl of membranal fraction lysate was pre-cleared with 50 μl of Sepharose-protein A beads (Sigma-Aldrich). hSST2-eYFP fusion protein was immunoprecipitated by incubating solubilized fractions overnight at 4 °C with a monoclonal anti-GFP antibody (Roche Applied Science) pre-bound to Sepharose-A beads. Immune complexes were collected by centrifugation, washed three times with buffer containing 30 mM Heps, 30 mM NaCl, and 0.1% Triton X-100, pH 7.5, and boiled for 5 min in 100 μl of 2X SDS sample loading buffer (100 μl Tris, pH 6.8, 4% SDS, 0.2% b-mercaptoethanol) before loading. Western blotting was performed with a Bio-Rad Page Bis-Trio electrophoresis system (Invitrogen). Cellular proteins were separated on a 4–12% Bis-Tris gel under reducing conditions, transferred to a polyvinylidene difluoride membrane, and immunoblotted with monoclonal anti-GFP antibody (Roche Applied Science) at 1:500 dilutions.

**Cell Fixation and Immunocytochemistry**—AtT-20 cells were plated in 12-well plates (106 cells/well) on lysis-coated 18-mm glass coverslips

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and transfected using Effectene (Qiagen) with the desired receptor-fluorescence construct (0.2 μg/well) 24 h prior to visualization. After 1 and 3 h of agonist treatment cells were washed with phosphate-buffered saline (pH 7.0), fixed in 4% paraformaldehyde for 2 h at room temperature, then washed with phosphate-buffered saline containing 0.1% BSA, dried, and mounted on a slide with Prolong Gold mounting medium (Molecular Probes, Eugene, OR). Cells transfected with 3×HA-hSST1, hSST2, hSST3, hSST4, and hSST5 were washed and treated with 0.1% Triton X-100, which was washed off 15 min later. Following 1-h incubation in phosphate-buffered saline containing 1% BSA, cells were stained with mouse monoclonal anti-hemagglutinin pre-conjugated with Alexa fluor 488 (Molecular Probes) overnight at 4 °C. Cells were then washed with phosphate-buffered saline-0.1% BSA, dried, and mounted on slides as described above.

Confocal Fluorescence Microscopy for Internalization Studies—Images were acquired with a Leica TCS SP confocal microscope. The 514 and 458 nm argon laser lines excited eYFP fusion proteins and eCFP, respectively. Fixed cells expressing tagged receptors were imaged with a narrow spectral detection window to reject auto-fluorescence. The pinhole was set to 1.0 Airy unit for best resolution. For live cells the confocal pinhole was set to 1.5 Airy unit, and a wide spectral detection window was used for maximum sensitivity. Acusto-optical-tunable-fil- ter was set to 30% to reduce bleaching. An image stack of 15 to 16 optical slices was recorded every 30 s. Movies were created by rendering maximal intensity projections of each time point into an uncompressed digital audio-visual-interleaved stream.

Statistical Analysis—ACTH, cAMP, and calcium oscillation amplitude analyses are expressed as mean ± S.E. percentage change from control within the same experiment, or as indicated. Sigmoid dose-response and two-site competition curves derived from non-linear regression analysis were used to assess ligand affinity, potency, and efficacy. Two-way ANOVA with Bonferroni analysis and paired t test were used to assess differences between and within treatment groups.

RESULTS

Agonists Binding Affinities in AtT-20 Cells—Binding affinities (Table I) of peptide analogs were measured in Chinese hamster ovary-K1 cell membranes transfected with hSST receptor subtypes and showed that SST2- and SST5-selective peptide agonists were specific for SST2 (300 pM) and SST5 (2 nM), respectively. Octreotide preferentially bound SST2, with moderate affinity for SST5, whereas SRIF-28 bound all SSTs, with highest affinities for both SST5 and SST2. Binding affinities of SST2-agonist ligands (including SST2-selective agonist, octreotide, and SRIF-28) to AtT-20 cell membranes were similar (200–700 pM). These affinities were also similar to those observed in hSST2-transfected Chinese hamster ovary cells. SST5 agonist binding affinity to AtT-20 membranes and to hSST5-transfected Chinese hamster ovary cells exhibited 50-fold lower affinity compared with SST2 agonists to SST2. The SST2 antagonist also bound hSST3 and hSST5, the latter at relatively high concentrations.

ACTH and cAMP Regulation—Forskolin (20 μM) induced ACTH secretion 3- to 5-fold and intracellular cAMP levels 10- to 15-fold (data not shown). In AtT-20 cells, dose-dependent inhibition curves of forskolin-stimulated ACTH secretion and cAMP accumulation are depicted in Fig. 1 (A and B, respectively), whereas their EC_{50} values are summarized in Table II and compared with those with SST2 antagonist co-treatment. Octreotide and the SST2-selective agonist exhibited similar potencies (EC_{50} values ranging from 20 to 76 pM) for both ACTH and cAMP inhibition. SRIF-28 exhibited the highest potency (EC_{50} 2 fM) of the ligands studied, inhibiting both forskolin-stimulated ACTH secretion and cAMP accumulation; the SST5-selective agonist exhibited the lowest potency. The SST2 antagonist alone (1 pM to 1 μM) did not alter cAMP levels.
Co-treatment with SST2 antagonist (500 nM) reduced SST2-selective agonist and octreotide potencies for cAMP and ACTH inhibition, and in particular SRIF-28 potency, causing a 200-fold decrease in EC50 values. Treatment with vehicle alone did not alter cAMP levels (data not shown).

The SST2-selective agonist, SST5 agonist, octreotide, and SRIF-28 did not differ in their efficacy (two-way ANOVA, Bonferroni p > 0.05), all partially suppressing ACTH and cAMP (Fig. 1).

**BSCOs in Resting AtT-20 Cells**—Unstimulated AtT-20 cells exhibited different baseline patterns of calcium oscillation. Prior to drug treatment ~70% of cells exhibited regular continuous oscillation patterns (509 of 717 cells visualized), whereas ~30% show short (arbitrarily set at ≤30 s or 100 frames) (154 cells) or long (arbitrarily set at >30 s or 100 frames) (54 cells) oscillation pauses. Cells showing long pauses prior to treatment were excluded from the analysis. Oscillations were dependent on the presence of calcium in the medium, because suspension in calcium-depleted medium significantly reduced oscillation amplitude and abolished ligand responses (data not shown). Two drug effect analyses were evaluated in parallel: 1) a graphic qualitative presentation of representative single cell dose-response curves and 2) a semi-quantitative analysis of multiple cells, to create an averaged dose-response curve and to determine potencies. BSCOs were observed in cell cytoplasm, and intensity values were averaged without the ability to assess oscillation origin (i.e. cell membrane, endoplasmic reticulum, mitochondria, or other cellular calcium stores). As a negative control, vehicle (10 μl) was added every 5 min to the suspension (1 ml) as cells were scanned. The vehicle did not significantly alter oscillation patterns (Fig. 2A), except for a gradual increase in average normalized oscillation amplitude (aNOA) of up to 35% after 50 min of scanning (Fig. 2B). Rapid BSCOs recovery was observed 5 min after drug treatment even though ligands were not washed out (data not shown). This pattern allowed us to avoid the need to wash out
the agonist from the medium, a procedure that causes cell detachment from the coverslip. Agonists were therefore added at 10-fold concentration increments every 5 min. This approach was established for the patch clamp technique (27, 44). Nimodipine, the L-type calcium channel blocker, abolished BSCOs (Fig. 3A) and reduced aNOA with an EC\textsubscript{50} of 9 nM and V\textsubscript{max} of 75% (Fig. 3B). Nimodipine also reduced forskolin-stimulated ACTH secretion by 30% (Fig. 3C) but did not alter forskolin-stimulated cAMP accumulation (Fig. 3C). Fig. 4A depicts dose-dependent BSCOs inhibition by the SST2-selective agonist as observed by initial increased amplitude accompanied by the appearance of long BSCOs pauses at concentrations as low as 1–10 fm. Thereafter, pauses gradually lengthened while amplitudes gradually decreased. These changes are reflected in the dose-response curve (Fig. 4B), with decreased aNOA occurring with an EC\textsubscript{50} of 900 fm (Table III), a ∼20-fold higher potency compared with adenylate cyclase inhibition (Table II). Fig. 4C portrays that SST2-selective agonist concentrations lower than 1 fm do not affect BSCOs, as reflected by unaltered aNOA, in contrast to that observed with SRIF-28.

The SST5-selective agonist induces brief oscillation pauses, concomitant with a significant and sustained increased amplitude (up to 2-fold), decreasing only at 1 μM ligand concentration, together with the appearance of long pauses (Fig. 5A). This phenomenon is reflected in the aNOA curve for the SST5-selective agonist, exhibiting a ∼50% increase from baseline values, and ∼40% over the matching phase in vehicle-treated cells. At very high concentrations, aNOA is decreased, although still not achieving baseline levels. The extrapolated EC\textsubscript{50} for BSCOs inhibition by the SST5-selective agonist is >1 μM (Table III), a dose >100-fold higher than its potency to inhibit adenylate cyclase (Table II).

Octreotide, the combined SST2 and SST5 agonist, induced an initial amplitude increase similar to the SST5-selective agonist with long pauses appearing at 100 pm (Fig. 6A). Together, the pattern resembles a combination of both SST2 and SST5 effects. This is also reflected in the dose-response curve for aNOA, with an EC\textsubscript{50} of 12 μM (Fig. 6B), −10-fold lower than the potency of the SST2-selective agonist (Table III).

Unlike the other agonists, SRIF-28 induced short BSCOs pauses at sub-femtomolar concentrations accompanied by amplitude increase, and long pauses at 10 pm (Fig. 7A). Notably, the amplitude does not return to the baseline level, but remains higher until 100 pm concentrations are achieved. This pattern is reflected in the dose-response curve for aNOA, whereby, unlike the other ligands, resembles a two-site competition curve (Fig. 7B). The first competition site represents an EC\textsubscript{50} at fm levels, similar to that for cAMP and ACTH inhibition potency, and a second competition site with an EC\textsubscript{50} at pm levels, equivalent to the EC\textsubscript{50} for octreotide (Table III).

Receptor Internalization—hSST2-eYFP (C-terminal tagging) and hSST5-eYFP (C-terminal tagging) structure and functionality were verified by several approaches: 1) sequences of both constructs were confirmed as: hSST2: 1110 bp; hSST5: 1095 bp; 2) the observed fusion protein size was, as expected a ∼80–95 kDa broad band of hSST2-eYFP, and a hSST5-eYFP ∼75–85 kDa (eYFP ∼25 kDa) (Fig. 8A); 3) SRIF-14 treatment of either hSST2- or hSST5-eYFP transfected forskolin-stimulated Chinese hamster ovary cells reduced intracellular cAMP levels (Fig. 8B); and 4) membranal localization of both fusion proteins was clearly visualized (Fig. 8, C to H). hSST2-eCFP internal-
ized in the same pattern as hSST2-eYFP. All cells have multiple membranal extrusions with minimal intracellular accumulation. A commercial fusion protein comprising hSST5 and 3XHA (N-terminal tagging) exhibited membranal localization similar to hSST5-eYFP localization. Addition of serum to the medium did not inhibit internalization, unlike its effect on ACTH secretion, cAMP accumulation, and baseline spontaneous calcium oscillation.

Treatment with SST2 agonists (SST2-selective agonist, octreotide, and SRIF-28) activated hSST2-eYFP internalization in AtT-20 cells (Fig. 9), as well as in human HEK-293, HeLa, and MCF-7 cells (data not shown). In contrast, neither hSST5-eYFP nor 3XHA-hSST5 internalized with agonist treatment by any of the ligands at concentration of up to 10 μM. Table IV depicts agonist concentrations that initiate SST2-eYFP internalization. Only the SST2-specific agonist induced internalization at 1 nm, whereas octreotide and SRIF-28 induced internalization at 10 nm. Co-treatment with SST2 antagonist (500 nM) completely blocked internalization in the concentrations studied. hSST2-eYFP did not internalize spontaneously or with SST2 antagonist treatment. In live cell analysis, co-expression of both receptors did not alter their respective internalization patterns; hSST2-eCFP rapidly internalized, whereas hSST5-eYFP remained localized to the membrane Fig. 10.

### DISCUSSION

Measuring BSCOs, receptor internalization, cAMP accumulation, and ACTH secretion in AtT-20 cells treated with highly selective receptor peptide agonists highlights the complexity of SST2 and SST5 regulation of corticotroph tumor cell function. The results summarized in Table V emphasize the need to evaluate responses to somatostatin analogs in both stimulated and resting cells. Integration of the results obtained from the four pathways studied suggests that SST5 and SST2 functions are interrelated and act together to control AtT-20 cell responses to SRIF. SST2 is a dominant regulator of corticotroph cell function, whereas SST5 modulates SST2 action.

Comparing binding affinities of SST2- and SST5-selective agonists to those of octreotide and SRIF-28 suggest that SST2 is the major contributor to AtT-20 cells membrane binding. As expected, forskolin-stimulated AtT-20 cells respond to each ligand with dose-dependent inhibition of cAMP accumulation and ACTH secretion. The potency of octreotide is reversed by the SST2 antagonist as is the SST2-selective agonist, without obviously evident SST5 agonism. The lower potency of the SST5-selective agonist is in keeping with the observed lower

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**FIG. 4. BSCOs in SST2-selective agonist-treated cells.** A, intensity graphs obtained from a representative AtT-20 cell scanned by confocal microscopy at constitutive 5-min intervals, for 45 min. The axes are as described for the untreated cell, and graphs are read from left to right. Only representative graphs are depicted. The first graph describes intensities obtained from the same cell prior to addition of lowest ligand concentration. SST2-selective agonist induces long pauses in BSCOs (>100 frames or 30 s) at concentration as low as 10 fM. B, the percent change of averaged normalized oscillation amplitude (aNOA) obtained from 54 cells treated with 10-fold increments of ligand concentrations (1 × 10⁻²⁰ M). The SST2-selective agonist decreased aNOA dose dependently (two-way ANOVA, a, p < 0.01; b, p < 0.001) as compared with untreated cells group scanned for 50 min. C, the percent change of aNOA obtained from 84 cells treated with 10-fold increments of ligand concentrations (log molar). The depicted dose-response curve is a combination of two experiments: 30 cells were exposed to ligand (0.0001 fM to 1 pm), and 54 cells were exposed to ligand (1 fM to 10 nm). The SST2-selective agonist decreased aNOA dose dependently (t test; a, p < 0.03; b, p < 0.0001) as compared with untreated cells in the same experiments. The SST2-selective agonist does not induce an initial aNOA increase.

**TABLE III**

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Potency derived from dose-response curve of aNOA for each ligand. SST2- and SST5-selective agonists and octreotide exhibit a single competition dose-response curve and single EC₅₀. SRIF-28 exhibits a biphasic dose-response curve with both high and low affinities.
binding affinity for SST5, as compared with the affinity of the SST2-selective agonist for SST2. Of the agonists studied, the multireceptor agonist SRIF-28 is the most potent inhibitor of cAMP accumulation and ACTH secretion, at femtomolar levels. Higher SRIF-28 versus SRIF-14 potency in AtT-20 cells was previously reported (45), although not to such an extent. The observed differences could be attributed to cell age, serum used, SRIF-28 purity, and disulfide bond stability in this molecule. The high affinity of SRIF-28 to SST5, as previously speculated (45), along with similar affinity to SST2, still does not explain the affinity:potency ratio of 100,000:1. Other explanations include SRIF-28 inhibition of other SSTs such as SST1, -3, or -4 that may be present in undetectable quantities in AtT-20 cells, even though no obvious binding of these subtypes in these cells has been shown (11). Alternative explanations include preferential induction of specific synergy between SST2 and SST5 by SRIF-28, and not by octreotide, recognition of different G proteins, or SRIF-28 inhibition of a yet unknown SST in these cells (46).
peptides, including SRIF (47, 48), corticotropin-releasing factor-like peptides sauvagine and urotensin-I (49), growth hormone releasing factor (50), and PACAP (51). These very low potencies support a role for SRIF-28 in subtle regulation of hormone/neurotransmitter secretion as part of homeostasis maintenance, utilizing an autocrine or paracrine mechanism of action (52) whereby relatively few peptide molecules are required to induce the desired biologic effect. The SST2 antagonist (500 nM) exhibited more prominent inhibition of SRIF-28 effects compared with its action on the SST2-selective agonist or octreotide. This is likely related to the ratio of agonist:antagonist concentrations. At 20 pM the SST2-selective agonist the ratio is 1:2.5 \times 10^4, whereas at 1 fm SRIF-28 the ratio is 1:10^9. The antagonist may also competitively inhibit both SST2 and SST5 SRIF-28-related effects, because the SST2 antagonist also binds SST5, although at higher concentrations.

A major contribution to understanding respective roles for SST2 and SST5 in AtT-20 cells is derived from analysis of calcium oscillations in AtT-20 cells. The resting BSCOs pattern did not change during 50-min scanning except for a moderate increase in amplitude, which is reflected in the aNOA curve. The reason for this observation is not clear, and may be explained by the prolonged laser exposure of the cells as previously suggested in other cell systems (53). The moderate increase in aNOA actually further emphasizes the inhibitory results obtained with the ligands, with every inhibition or stimulation likely being stronger than the actual aNOA observed. Because somatostatin receptors reduce intracellular calcium levels in AtT-20 cells mainly through L-type calcium channels (23, 27), nimodipine, the competitive L-type channel blocker, is an appropriate positive control for evaluating agonist effect. The EC_{50} for nimodipine inhibition of calcium currents through L-type calcium channels (187 nM) was previously described in seven stimulated rat corticotroph cells in primary culture (54). We show here that an EC_{50} of 9 nM with maximal 75% inhibition of BSCOs in mouse AtT-20 corticotroph cells, but no effect on baseline or stimulated cAMP and only 30% reduction of stimulated ACTH, suggesting that somatostatin-induced blockage of L-type calcium channels in AtT-20 cells is not required for suppressing adenylyl cyclase activity, but is necessary for partially blocking ACTH secretion, presumably by blocking exocytosis (55, 56).

SST2 and SST5 exhibit differential regulation of calcium channels in AtT-20 cells. This notion was previously suggested through L-type calcium channel coupling of SST2 and SST5, which desensitizes SST5 but not SST2, suggesting distinct mechanisms for the two subtypes (27). The SST2 effect is marked by long BSCOs pauses appearing at concentrations \geq 1 \mu M likely relate to the SST2 concomitant effect of the SST5-selective agonist, because at these concentrations SST5-selective agonist is 10-fold beyond its half-maximal binding affinity to SST2.
Both selective agonists induce increased amplitude, but the SST5 increase is more prominent as evidenced by the dose-response curves for aNOA. The combination of these two different subtype-selective properties in octreotide result in a shift to the right of the BSCOs inhibition as seen by higher EC50 and pauses appearing at higher concentrations compared with those achieved for the SST2-selective agonist. This pathway exhibits an SST5-regulating effect on SST2.

SRIF-28 exhibits an SST5 effect already at sub-femtomolar concentrations and an SST2 effect at picomolar concentrations. The difference between SRIF-28 and the other ligands is the yet unexplainable femtomolar concentration at which the aNOA decrease to pretreatment levels, and cAMP accumulation and ACTH secretion is half-maximally decreased. Assuming that there are, as reported in the literature (10, 11), solely SST2 and SST5 receptors expressed in AtT-20 cell membranes, and SRIF-28 has equal affinity to both receptors, then the first phase of the aNOA dose-response curve is likely a reflection of

---

**Fig. 8.** **Characterization of SST2-eYFP and SST5-eYFP fusion proteins.** A, depicts transfected AtT-20 cell protein extract was immunoprecipitated and immunoblotted with anti-GFP antibody. Western blot exhibited an expected broad ~80–95 kDa band of SST2-eYFP fusion protein and a 75–85 kDa band of SST5-eYFP. The 55- and 25-kDa bands observed on the gel likely represent IgG heavy and the GFP protein. B, depicts receptor subtype-transfected Chinese hamster ovary cells treated with either forskolin (15 μM) or co-treated with forskolin and SRIF-14. SST2 or SST5 expression in these cells inhibited cAMP levels when cells were treated with SRIF-14. C–H demonstrate membranal trafficking of both fusion receptor proteins. Images on the left are an overlay of 200-nm cell sections, and on the right are cross-sections of the same cells transfected with SST2-eYFP (C and D; upper panel), SST5-eYFP (E and F; middle panel), 3XHA-hSST5 (G and H; lower panel). Cells were transfected on poly-l-lysine-coated coverslips, fixed, and analyzed by fluorescent confocal microscopy. All fusion receptors traffic to the membrane.

**Fig. 9.** **SST2-selective agonist induces SST2-eYFP internalization in AtT-20 cells at 1 nM concentration.** Cells were plated on poly-l-lysine-coated coverslips, transfected with SST2-eYFP, and treated with increasing ligand concentrations. Cells were fixed and visualized by fluorescent confocal microscopy. The images depict an overlay of a series of cell sections and are arranged from left to right: A, untreated cell shows membranal distribution of the SST2-eYFP receptor with multiple membranal extrusions. B, treatment with SST2-selective agonist (1 nM) induces internalization. C, intensification of internalization with 10 nM ligand.
Somatostatin Receptors and ACTH

AtT-20 cells were plated and grown as described. Following 1-h treatment with increasing doses of ligand with or without SST2 antagonist, cells were washed, fixed, mounted, and visualized by confocal microscopy. SST2 agonist activated internalization of hSST2-eYFP at 1 nM, whereas octreotide and SRIF-28 activated internalization from 10 nM and higher. Co-treatment with SST2 antagonist (500 nM) blocked internalization.

**TABLE IV**

**Receptor internalization**

AtT-20 cells were plated and grown as described. Following 1-h treatment with increasing doses of ligand with or without SST2 antagonist, cells were washed, fixed, mounted, and visualized by confocal microscopy. SST2 agonist activated internalization of hSST2-eYFP at 1 nM, whereas octreotide and SRIF-28 activated internalization from 10 nM and higher. Co-treatment with SST2 antagonist (500 nM) blocked internalization.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Preferred receptor subtype(s)</th>
<th>SST2-eYFP internalization, agonist concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>100 pM + antag.</td>
</tr>
<tr>
<td>SRIF-28</td>
<td>SST1 to 5</td>
<td>n</td>
</tr>
<tr>
<td>BIM-23120</td>
<td>SST2</td>
<td>n</td>
</tr>
<tr>
<td>BIM-23206</td>
<td>SST5</td>
<td>n</td>
</tr>
<tr>
<td>Octreotide</td>
<td>SST2, -5</td>
<td>n</td>
</tr>
<tr>
<td>BIM-23454</td>
<td>SST2 antagonist</td>
<td>n</td>
</tr>
</tbody>
</table>

**"+ antag." represents co-treatment with agonist and SST2 antagonist. n, no; Y, yes.**

**TABLE V**

**Summary of affinity, potency, and internalization for SST2- and SST5-selective agonists, octreotide, and SRIF-28 in AtT-20 cells**

<table>
<thead>
<tr>
<th></th>
<th>Binding in resting cells</th>
<th>Forskolin stimulation</th>
<th>Calcium oscillations in resting cells</th>
<th>Internalization in resting cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>AtT-20</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>ACTH</td>
</tr>
<tr>
<td></td>
<td>AtT-20</td>
<td>200 pm</td>
<td>10 nm</td>
<td>300 pm</td>
</tr>
<tr>
<td></td>
<td>Octreotide</td>
<td>100 fm</td>
<td>1 μM</td>
<td>100 fm</td>
</tr>
<tr>
<td></td>
<td>SRIF-28</td>
<td>10 pm</td>
<td>2 fm</td>
<td>10 pm</td>
</tr>
</tbody>
</table>

"N/A, not ascertainable.

SST5 (an increase in aNOA, increased amplitude, brief pauses, and return to pretreatment levels at the first EC<sub>50</sub>), whereas the second phase reflects the SST2 effect (as seen with octreotide). SRIF-28 exhibits SST5 affinity ~5-fold higher than that for the SST5-selective agonist and according to the literature may be even higher (57). Because the SST5-selective agonist affects BSCOs already at 100 fm, the SRIF-28 high-affinity SST5 component would be expected to initiate its action at extremely low concentrations.

However, the low SST5 affinity in AtT-20 cell membranes may not favor such a hypothesis. SRIF-28 super-potency may yet derive from other mechanisms as mentioned above.

**FIG. 10. SRIF-28 treatment of AtT-20 cells co-expressing hSST2-eCFP and hSST5-eYFP.** AtT-20 cells were plated and transfected as described under "Materials and Methods." Cells were placed in the confocal microscope incubator chamber, scanned for 3 min (‘0’ depicts image taken after 3 min of scanning without treatment), after which SRIF-28 (10 nM) was added to the medium; thereafter cells were scanned every 3 min. Images are presented as an overlay of all cell sections for each receptor alone (hSST2-eCFP: upper panel; hSST5-eYFP: middle panel) and for both receptors (lower panel). Rapid internalization of hSST2-eCFP was observed at 3 min (‘3’) gradually intensifying. hSST5-eYFP remained localized to the membrane.

**TABLE V**

**Summary of affinity, potency, and internalization for SST2- and SST5-selective agonists, octreotide, and SRIF-28 in AtT-20 cells**

<table>
<thead>
<tr>
<th></th>
<th>SST2 agonist</th>
<th>SST5 agonist</th>
<th>Octreotide</th>
<th>SRIF-28</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AtT-20</td>
<td>AtT-20</td>
<td>Octreotide</td>
<td>SRIF-28</td>
</tr>
<tr>
<td></td>
<td>200 pm</td>
<td>10 nm</td>
<td>300 pm</td>
<td>700 pm</td>
</tr>
<tr>
<td></td>
<td>100 pm</td>
<td>1 μM</td>
<td>100 pm</td>
<td>10 pm</td>
</tr>
<tr>
<td></td>
<td>10 nm</td>
<td>10 nm</td>
<td>10 nm</td>
<td>10 nm</td>
</tr>
</tbody>
</table>

"N/A, not ascertainable."
Internalization studies further support the notion that SST5 modulates SST2 actions. Octreotide and SRIF-28, both containing an SST5 agonist component, initiate internalization at doses 10-fold higher than the SST2-selective agonist. This observation may imply that SST5 binding and activation attenuates SST2 internalization. Moreover, using two differently tagged receptors, SST5 (either C- or N-terminally tagged) was shown not to internalize with any ligand, but constantly localize to the membrane in both mouse and human cell lines. Rapid internalization of the membranal “high turnover” SST2 may explain the observation that SST2 exhibits a spare receptor phenomenon, whereas SST5 does not. The higher concentrations at which the receptor internalizes compared with respective ligand potencies may not necessarily reflect in vivo behavior as these are human receptors transfected into mouse cells. However, the 95% homology between human and mouse SST2 (57) and the observation that similar concentrations for internalization were also observed in human cells, strengthen the relevance of the results. SST5, as previously reported for SST1 (31, 33), does not internalize, in agreement with previous reports suggesting either sub-membranal localization or enhanced trafficking to the membrane of SST5 after treatment with SRIF-14 (33, 38). An effect caused by the difference between human and mouse SST5 is possible; however, SRIF-28 was shown to bind mouse or human transfected SST5 with the same affinity (competing [125I]LT- SRIF-28) (58, 59), human and mouse SST5 share 80% homology, and we also have demonstrated that hSST5 did not internalize in human cells.

The results suggest functional interaction between SST2 and SST5 in AtT-20 cells. Although SST2 is the dominant activator for SRIF in these cells, SST5 serves as a regulator of SST2 action, attenuating the potency of SST2 agonists, and retaining for SRIF in these cells, SST5 serves as a regulator of SST2 action, attenuating the potency of SST2 agonists, and retaining for SRIF in these cells, SST5 serves as a regulator of SST2 action, attenuating the potency of SST2 agon...
Somatostatin Receptor Type 5 Modulates Somatostatin Receptor Type 2 Regulation of Adrenocorticotropic Secretion
Anat Ben-Shlomo, Kolja A. Wawrowsky, Irina Proekt, Nathaniel M. Wolkenfeld, Song-Guang Ren, John Taylor, Michael D. Culler and Shlomo Melmed

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