

Receptor Signaling and Endocytosis Are Differentially Regulated by Somatostatin Analogs

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ABSTRACT

Upon hormone stimulation, the sst2 somatostatin receptor couples to adenylyl cyclase through $G_{i/o}$ proteins and undergoes rapid endocytosis via clathrin-coated pits. In this study, we determined the relationship between the ability of ligands to induce sst2 receptor internalization and inhibit adenylyl cyclase. Immunocytochemical studies demonstrated that peptide agonists [such as somatostatin-14, cortistatin-17, octreotide, vapreotide, KE108 (Tyr0-cyclo[D-diaminobutyric acid-Arg-Phe-Phe-D-Trp-Lys-Thr-Phe)], and SOM230 (cyclo[diaminoethylcarbamoyl-hydroxyproline-phenylglycine-D-Trp-Lys-(4-O-benzyl)-L-Tyr-Phe)] and nonpeptide agonists (such as L-779,976), stimulated the rapid endocytosis of sst2 receptors in human embryonic kidney 293 and CHO-K1 cells. In contrast, two antagonists did not induce receptor endocytosis by themselves and completely blocked agonist stimulation. Using a quantitative enzyme-linked immunosorbent assay to measure sst2 receptor sequestration, we found that peptide agonists varied by

more than 100-fold in their potencies but exhibited the same efficacy as somatostatin-14. In contrast, L-779,976 did not induce maximal receptor internalization. It is interesting that although β arrestin-2 was recruited to cell surface sst2 receptors after stimulation with either somatostatin-14 or L-779,976, the β arrestin-receptor complex dissociated earlier in the endocytic pathway with the nonpeptide ligand. Although all agonists, including L-779,976, produced the same maximal inhibition of cyclic AMP, the potency ratio for inhibition of cyclic AMP and stimulation of receptor endocytosis varied by 15-fold. In general, native peptides showed similar potencies for cyclic AMP inhibition and receptor endocytosis, whereas short therapeutic analogs were substantially more potent at inhibiting cyclic AMP synthesis. These results demonstrate that the activity of somatostatin analogs to regulate receptor endocytosis and signaling are not tightly linked and provide compelling evidence for the induction of agonist specific states of the sst2 receptor.

Somatostatin is a regulatory peptide that exerts a broad spectrum of actions in endocrine, neuroendocrine, neuronal, smooth muscle, and immune cells (for review, see Schonbrunn, 2001, 2004; Olias et al., 2004). These actions include modulation of neurotransmission in the central and peripheral nervous system, inhibition of hormone secretion by the pancreas and the pituitary, inhibition of exocrine secretion in

the pancreas and the gastrointestinal tract, and regulation of smooth muscle contraction. Moreover, somatostatin has been shown to inhibit secretion and growth by a number of neuroendocrine tumors (Reubi, 1997). The biological actions of somatostatin are mediated by six G protein-coupled receptors encoded by five genes, named *sst1* through *sst5*. The sst2 receptor exists in two variants in rodents: the unspliced sst2A form and the spliced sst2B form with a different carboxyl terminus. So far, however, only the sst2A variant has been found in humans.

The sst2 receptor is the most widely distributed somatostatin receptor subtype in both normal tissues and tumors and hence has been aggressively targeted pharmacologically.

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ABBREVIATIONS: CHO, Chinese hamster ovary; HEK, human embryonic kidney; M6PR, mannose 6-phosphate receptor; HA, hemagglutinin; SMS 201-995, octreotide; TOC, [Tyr³]octreotide; RC-160, vapreotide; GFP, green fluorescent protein; FBS, fetal bovine serum; PS, 100 mM phosphate buffer containing 0.15 M sucrose; BSA, bovine serum albumin; F12LH, Ham's F-12 medium containing 5 mg/ml lactalbumin hydrolysate and 20 mM HEPES, pH 7.4; TBS, Tris-buffered saline; ELISA, enzyme-linked immunosorbent assay; IBMX, 3-isobutyl-1-methylxanthine; TGN, trans-Golgi network; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; SS-14, the 14 amino acid form of somatostatin; SS-28, the NH₂-terminally extended 28 amino acid form of somatostatin.

Based on studies with selective somatostatin analogs as well as knockout mice, the physiological effects mediated by the sst2 receptor include inhibition of growth hormone, glucagon, and gastric acid secretion (Viollet et al., 2000; Schonbrunn, 2001, 2004; Olias et al., 2004). Its broad but specific distribution in the central nervous system further suggests important modulatory effects on learning, memory, and motor control (Csaba and Dournaud, 2001).

Two properties of sst2 receptors have been exploited clinically. First, the effect of somatostatin analogs to inhibit secretion by a variety of neuroendocrine tumors (including carcinoids, vasoactive intestinal peptide-producing tumors, and pituitary tumors) is the primary reason these compounds are used therapeutically (Lamberts et al., 2002; Hofland and Lamberts, 2003; Reubi, 2003). This inhibitory effect on secretion results from receptor activation of pertussis-toxin sensitive G proteins that subsequently inhibit adenylyl cyclase, close voltage-sensitive calcium channels, and open specific potassium channels (Schonbrunn et al., 1996). Second, the sst2 receptor, like many other G protein-coupled receptors (GPCRs), is rapidly internalized after agonist-induced receptor phosphorylation (Hipkin et al., 1997). Receptor-mediated ligand endocytosis has been used clinically to catalyze the accumulation of stable, radiolabeled somatostatin analogs in tumor cells that express the sst2 receptor (Hofland and Lamberts, 2003; Reubi, 2003). Such radioligand accumulation allows receptor-expressing tumors and their metastases to be imaged by γ camera scintigraphy (Breeman et al., 2001) and to be treated by peptide receptor-targeted radiotherapy (Reubi, 2003).

A large number of stable peptide and nonpeptide ligands have been developed to pharmacologically target the sst2 receptor (Weckbecker et al., 2003). Some of the agonists are highly specific for the sst2 receptor subtype, whereas others, like the native peptides, are able to activate multiple sst receptors with little selectivity. Analogs currently used for the treatment of acromegaly and gastroenterological tumors include octreotide and similar short, stable peptides that bind to sst2 with high affinity and to sst3 and sst5 with lower affinity. Although there are few sst receptor subtype-selective antagonists, such compounds have now been identified (Bass et al., 1996; Rajeswaran et al., 2001). Nevertheless, there are no systematic studies to quantitatively compare the effects of somatostatin analogs on signaling and endocytosis. Such studies may not only identify compounds optimally targeted for tumor inhibition or tumor visualization, they could distinguish between different models for receptor function (Watson et al., 2000; Kenakin, 2003). If a direct proportionality exists between agonist strength to regulate second messenger formation and to induce receptor endocytosis, as previously reported for β_2 -adrenergic and muscarinic receptors (January et al., 1997; Szekeres et al., 1998; Edwardson and Szekeres, 1999), then the same activated receptor conformation is likely to be responsible for both actions. However, if the potency ratio or efficacy for signaling and receptor internalization vary for different agonists, then ligand specific receptor conformations are likely to exist. The latter conclusion has been reported for μ -, δ -, and κ -opioid receptors (Borgland et al., 2003; Li et al., 2003; von Zastrow et al., 2003) (for review, see Kenakin, 2003).

In the present work, we have characterized the effects of 11 ligands in CHO-K1 cells and HEK293 cells, two model cell

lines that have been used extensively for studies of receptor endocytosis (Menard et al., 1997). Furthermore, we quantitatively compared the potencies of these compounds to inhibit cyclic AMP synthesis and to induce receptor endocytosis, assays that are both performed in intact cells under similar incubation conditions. The agonists selected provide a 200-fold range in potencies to inhibit cyclic AMP formation and include both nonpeptide and peptide ligands. Finally, we examine the effect of two ligands with different efficacies for receptor internalization on β arrestin recruitment to and trafficking with the sst2 receptor.

The studies presented here show that the relative potencies of agonists to regulate cAMP production and receptor endocytosis vary substantially, demonstrating that ligand-specific conformations must exist for the sst2 receptor. Furthermore, some compounds exhibit lower efficacies for receptor internalization than for signaling to adenylyl cyclase, suggesting that such ligands could have different therapeutic profiles than the somatostatin analogs currently used clinically. These results indicate that, depending on the clinical requirements, it may be possible to generate sst2 analogs with activity profiles selective for receptor internalization or for signaling via pertussis toxin-sensitive G proteins.

Materials and Methods

Reagents. All reagents were of the best grade available and were purchased from common suppliers. The mouse monoclonal mannose 6-phosphate receptor (M6PR) antibody, recognizing the human cation-independent M6PR, was purchased from Abcam Limited (Cambridge, UK). The R2-88 antibody to the sst2A receptor was generated as described previously and has been extensively characterized (Gu and Schonbrunn, 1997; Reubi et al., 1998). Rabbit polyclonal and mouse monoclonal HA epitope antibodies were purchased from Covance (Berkeley, CA). The secondary antibodies Alexa Fluor 488 goat anti-rabbit IgG (H+L) and Alexa Fluor 546 goat anti-mouse IgG (H+L) were from Molecular Probes, Inc. (Eugene, OR), the Cy-2 goat anti-mouse IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA), and the horseradish peroxidase-conjugated goat anti-rabbit IgG was from Bio-Rad Laboratories, Inc. (Hercules, CA). 2,2-Azino-di[3-ethylbenzthiozinesulfonate-(6)] was purchased from Roche Diagnostics Corporation (Indianapolis, IN). Oligonucleotide primers were synthesized by Sigma-Genosys (The Woodlands, TX).

Peptides. Peptides were obtained as follows (see Table 1 for structures): SS-14 and SS-28 were synthesized at the Salk Institute or purchased from Bachem California (Torrance, CA), human Cortistatin-17 (de Lecea et al., 1996) was from the Salk Institute or Phoenix Pharmaceuticals, Inc. (Belmont, CA), octreotide (SMS 201-995) (Bauer et al., 1982) and [Tyr³]octreotide (TOC) (Reubi, 1984) were from Novartis Inc. (Basel, Switzerland), vapreotide (RC-160) (Cai et al., 1986) was purchased from Calbiochem (Läufelfingen, Switzerland) or Phoenix Pharmaceuticals, Inc. L-779,976 (Rohrer et al., 1998) was a gift from Merck Pharmaceuticals (Rahway, NJ), CYN-154806 (Bass et al., 1996; Nunn et al., 2003), Coy-14 (Rajeswaran et al., 2001), KE108 (Reubi et al., 2002), sst3-ODN-8 (Reubi et al., 2000), and CH-288 (Liapakis et al., 1996) were all synthesized at the Salk Institute. Peptides synthesized at the Salk Institute were provided by Jean Rivier.

Plasmid Construction. To generate the HA-sst2A plasmid, a 30-amino acid sequence containing three tandem repeats of the HA epitope and a three-amino acid linker (YPYDVDPDYA YPYDVDPDYA YPYDVDPDYA DLE) was inserted after the methionine residue at the extracellular amino terminus of the rat sst2A receptor using the polymerase chain reaction. The product, which encoded the N-terminally extended sst2A receptor, was subcloned into the pcDNA3.0

vector (Invitrogen, Carlsbad, CA), sequenced to confirm its accuracy, and used to transfect CHO-K1 cells. To generate the β arrestin-2-GFP plasmid, the rat β arrestin-2 gene was excised from a plasmid generously provided by Dr. Marc Caron (Duke University, Durham, NC) using BamHI and SacI and inserted into the pEGFP-N3 vector (BD Biosciences Clontech, Palo Alto, CA). The resulting construct encoded a hybrid protein with enhanced GFP attached to the carboxyl terminus of β arrestin-2.

Cell Lines. The clonal CHO-K1 cell line expressing the HA-epitope tagged rat sst2A receptor (CHO-sst2) was generated by transfection of CHO-K1 cells with the 3xHA-sst2A pcDNA3.0 plasmid using FuGENE6 (Roche Diagnostics). Stable transfectants were selected with 750 μ g/ml G418 and then a cell line, CHO-sst2, was isolated by dilutional cloning. CHO-sst2 cells were cultured in Ham's F-12 medium containing 10% fetal bovine serum (FBS) and 250 μ g/ml G418 (Invitrogen) at 37°C and 5% CO₂. The HEK293 cell lines expressing T7-epitope tagged human sst1 (HEK-sst1) or the T7-epitope tagged human sst2A (HEK-sst2) receptors were kindly provided by Dr. S. Schulz (Magdeburg, Germany), and were cultured at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium containing 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin and 500 μ g/ml G418 (Invitrogen). Saturation binding assays, carried out according to previously published procedures (Brown et al., 1990; Elberg et al., 2002) with [¹²⁵I]-[Tyr³]octreotide as the radiolabel, gave K_d values of 0.093 ± 0.026 nM and 0.048 ± 0.026 nM with CHO-sst2 and HEK-sst2 membranes, respectively. The measured receptor density was 3.0 ± 0.2 pmol/mg of CHO-sst2 membrane protein and 8.9 ± 0.5 pmol/mg of HEK-sst2 membrane protein.

Immunofluorescence Microscopy. HEK-sst2 cells were grown on 35-mm four-well plates (Cellstar; Greiner Bio-One GmbH, Frickenhausen, Germany) coated with poly-D-lysine (10 μ g/ml; Sigma-Aldrich, St. Louis, MO). Cells were treated with various analogs at 37°C in growth medium for the indicated times, and then rinsed twice with PS (100 mM phosphate buffer containing 0.15 M sucrose). After fixation and permeabilization for 7 min with ice-cold methanol (−20°C), cells were rinsed twice with PS, and blocked for 60 min at room temperature with PS containing 0.1% BSA. The cells were subsequently incubated for 60 min at room temperature with an sst2A-specific primary antibody (R2-88; Gu and Schonbrunn, 1997) diluted 1:1000 in PS and then washed three times for 5 min each with PS containing 0.1% BSA. The cells were then incubated for 60 min at room temperature in the dark with the secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (H+L) diluted in PS (1:600). Thereafter, the cells were washed three times for 5 min each with PS containing 0.1% BSA, embedded with PS/glycerol 1:1, and covered with a glass coverslip. The cells were imaged using a DM RB immunofluorescence microscope (Leica, Wetzlar, Germany) and a DP10 camera (Olympus, Tokyo, Japan).

To investigate the intracellular localization of the internalized sst2, we examined its colocalization with the M6PR, a marker for the *trans*-Golgi network (TGN)/late endosomal compartment. HEK-sst2 cells were incubated with or without 1 μ M SS-28 for 20 min at room

temperature, and then fixed, permeabilized, and blocked as described above. The cells were subsequently incubated for 60 min at room temperature with the sst2A-specific rabbit antibody R2-88 (1:1000 in PS) together with the monoclonal M6PR antibody (5 μ g/ml in PS). The cells were washed and then incubated sequentially with Alexa Fluor 488 goat anti-rabbit IgG (H+L) diluted in PS (1:600) and then, after further washing, with the Alexa Fluor 546 goat anti-mouse IgG (H+L) diluted in PS (1:400), each for 60 min at room temperature in the dark. The cells were then embedded as described above and imaged using a Zeiss Axioskop 2 immunofluorescence microscope and a Zeiss AxioCam HR camera. The program AxioVision version 4.1 was used to obtain the overlay pictures.

CHO-sst2 cells were grown for 2 days on 35-mm four-well plates (Cellstar; Greiner Bio-One GmbH) in Ham's F-12 medium supplemented with 10% FBS. On the day of the experiment, cell surface receptors were labeled by incubating cells for 2 h either at room temperature or on ice with the anti-HA monoclonal antibody (1:1000; Covance) diluted in Ham's F-12 medium containing 5 mg/ml lactalbumin hydrolysate and 20 mM HEPES, pH 7.4 (F12LH). After removing the unbound antibody, cells were washed with warm F12LH and incubated for 30 min at 37°C without or with analogs added for the times specified. Cells were then fixed and permeabilized for 2 min in −20°C methanol. After washing with Tris-buffered saline (TBS; 50 mM Tris and 150 mM NaCl, pH 7.4), cells were incubated with goat anti-mouse Cy-2 antibody (1:100; Jackson ImmunoResearch, West Grove, PA) in TBS for 1 h at room temperature. After three additional washes with TBS, the cells were embedded in Slowfade Light Antifade mounting solution (Molecular Probes, Eugene, OR) and covered with 11-mm glass coverslips. Cells were imaged using an Optiphot epifluorescence microscope (Nikon, Inc., Melville, NY) equipped with a 40 \times objective and a green fluorescence filter. Images were acquired with a digital camera and MagnaFire software (Optonics, Goleta, CA) on a MacIntosh G4 computer (Apple Computer, Cupertino, CA).

No immunostaining was observed in HEK-sst1 cells with R2-88 (1:1000 dilution) or in HEK-sst2 cells with R2-88 (1:1000) preabsorbed with 100 nM antigen peptide, consistent with the known specificity of this receptor antibody (Gu and Schonbrunn, 1997; Reubi et al., 1998). Likewise, no reactivity was observed in untransfected CHO-K1 cells with the HA antibody (1:1000). The time course for internalization observed here for the HA-epitope-tagged sst2A receptor immunostained with HA antibody was indistinguishable from that previously reported for the wild-type sst2A receptor immunostained with the R2-88 receptor antibody (Liu et al., 2003). Thus, the HA epitope tag did not affect receptor endocytosis. Furthermore, minimal receptor endocytosis was observed when the cells were incubated in the absence of agonist, demonstrating that the prebound HA antibody did not induce sst2 receptor internalization by itself.

Confocal Microscopy and β Arrestin-GFP Translocation Assays. CHO-sst2 cells were seeded at a density of 200,000 cells/well onto 18-mm glass coverslips in 12-well plates. After 24 h, cells were

TABLE 1
Structures of somatostatin analogs

Agonists	
SS-14	Ala-Gly-cyclo-[Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys]
SS-28	Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys-(SS-14)
Cortistatin-17	Asp-Arg-Met-Pro-cyclo-[Cys-Arg-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Ser-Ser-Cys]Lys
Vapreotide	D-Phe-cyclo-[Cys-Tyr-D-Trp-Lys-Val-Cys]-Trp-NH ₂
Octreotide	D-Phe-cyclo-[Cys-Phe-D-Trp-Lys-Thr-Cys]-Thr-ol
[Tyr ³]Octreotide	D-Phe-cyclo-[Cys-Tyr-D-Trp-Lys-Thr-Cys]-Thr-ol
KE108	Tyr-cyclo-[D-Dab-Arg-Phe-Phe-D-Trp-Lys-Thr-Phe]
SOM230	cyclo-[Dec-HyPro-Phe-D-Trp-Lys-Tyr(Bzl)-Phe]
Antagonists	
Coy-14	Cpa-cyclo-[D-Cys-Tyr-D-Trp-N-methyl-Lys-Thr-Cys]-Nal-NH ₂
CYN-154806	Acetyl-4NO ₂ -Phe-cyclo-[D-Cys-Tyr-D-Trp-Lys-Thr-Cys]-D-Tyr-NH ₂

Cpa, 3-(4-chlorophenyl)alanine; Dab, diaminobutyric acid; Dec, diaminoethylcarbamoyl; HyPro, hydroxyproline; Nal, 3-(2-naphthyl)alanine; Phg, phenylglycine; Tyr(Bzl), (4-O-benzyl)-L-tyrosine.

transfected with 0.5 μg of β arrestin-2-GFP using Eugene 6 (Roche Diagnostics Corporation). Twenty-four hours after transfection, cells were incubated with mouse anti-HA antibody (1:1000) for 2 h on ice in F12LH medium to label cell surface receptors and then treated at 37°C for the indicated times with a 100 nM concentration of either SS-14 or L-779,976. Cells were then chilled and fixed with 4% paraformaldehyde for 20 min at room temperature. After washing with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS at 4°C for 30 min and then incubated with Texas Red-conjugated goat anti-mouse antibody (1/200; Jackson ImmunoResearch) in PBS for 1 h at room temperature. After further washing with PBS, coverslips were mounted using SlowFade mounting medium (Molecular Probes, Inc., Eugene, OR). Confocal microscopy was performed on a Zeiss laser-scanning confocal microscope (LSM-510) in multitrack mode using excitation at 488 nm and 543 nm. Enhanced GFP and Texas Red were detected using band-pass filters at 500 to 530 nm and long-pass filters at 585 nm, respectively. Images were processed using Photoshop (Adobe Systems, Mountain View, CA). Cells shown are representative of the majority of cells observed.

Quantitative Assay for Receptor Internalization. Receptor internalization was measured using ELISA to quantitate HA-epitope-tagged sst2 on the cell surface. CHO-sst2 cells were seeded in 24-well plates in Ham's F-12 medium containing 10% fetal bovine serum without G418 and cultured for 2 to 3 days. On the day of the assay, cells were incubated with rabbit anti-HA antibody (1:1000) for 2 h at room temperature in F12LH medium to label cell surface receptors. After washing with F12LH to remove unbound antibody, cells were incubated for 30 min at 37°C either without or with ligands added for the times indicated. Incubations were terminated by placing plates in an ice bath. Cells were then washed twice with ice-cold PBS and fixed for 10 min at room temperature with 3% paraformaldehyde in PBS, pH 7.4. Nonspecific binding sites were blocked by incubating cells for 30 min at room temperature with PBS containing 1% bovine serum albumin (BSA; Fraction V, Sigma-Aldrich Co., St. Louis, MO). Cells were then incubated for 60 min at room temperature with goat anti-rabbit IgG horseradish peroxidase conjugate (1:1000) in blocking buffer. After three additional washes with PBS, antibody binding was measured by adding 0.3 ml of horseradish peroxidase substrate (2,2'-azino-di[3-ethylbenzthiozolesulfonate-(6)]). The OD_{405} was measured after a 45-min incubation at room temperature. Surface receptor remaining after ligand treatment was calculated as the absorbance measured in treated cells expressed as a percentage of the absorbance in untreated cells. Nonspecific absorbance, determined in experiments in which CHO-sst2 cells were incubated without either HA antibody or secondary antibody, or nontransfected CHO-K1 cells were incubated with both antibodies, was less than 1% of experimental values. Furthermore, absorbance varied linearly with the number of sst2A receptor-expressing cells in a dish, demonstrating that product formation in the ELISA was proportional to surface sst2A receptors. Figures show the results of a single experiment, with each point representing the mean \pm S.E.M. of two wells assayed in triplicate ($n = 6$). All experiments were repeated at least twice.

Measurement of Intracellular cAMP Accumulation. CHO-sst2 cells were seeded at a density of 100,000 cells/well in 12-well plates and grown for 4 to 5 days at 37°C in Ham's F-12 medium supplemented with 10% FBS. On the day of the experiment, cells were incubated for 3 h at 37°C with 3.5 $\mu\text{Ci}/\text{ml}$ [^3H]adenine (24 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) in F12LH. Cells were then washed once to remove the unincorporated [^3H]adenine, preincubated with the phosphodiesterase inhibitor 1 mM 3-isobutyl-1-methylxanthine (IBMX) for 5 min, and then treated for 10 min at 37°C with somatostatin receptor ligands in the presence of 10 μM forskolin (Calbiochem, San Diego, CA) and 1 mM IBMX. The medium was removed, and the reaction was terminated by the addition of 1 ml of ice-cold stop buffer (5% trichloroacetic acid, 0.1 mM unlabeled cAMP, and 20 mM ATP). [^{32}P]cAMP (approximately 5000 cpm in 100 μl of water) was then added to provide an

internal control for recovery. The amount of [^3H]cAMP formed in the assay was determined by sequential chromatography through Dowex 50 and neutral alumina columns as described previously (Salomon, 1991). Each point represents the mean \pm S.E.M. of triplicate wells.

Statistical Analysis and Curve Fitting. Figures show data expressed as the mean \pm S.E.M. from a single experiment and are representative of at least two different experiments. The half-times for agonist-induced receptor internalization were obtained by fitting data to a single exponential decay curve. Values for EC_{50} were calculated using Prism (ver. 4.0; GraphPad Software, San Diego, CA) by least-squares nonlinear regression analysis of dose-response curves fit to a one-component sigmoidal curve with a Hill coefficient of -1 .

Results

Immunocytochemical Characterization of sst2 Receptor Internalization. Both the rate and the extent of sst2 receptor internalization varied widely in previous studies. However, these investigations used different cell types for receptor expression and lacked consistency in the ligands used to drive sst2 endocytosis (Hukovic et al., 1996; Hipkin et al., 1997; Nouel et al., 1997; Roosterman et al., 1997; Roth et al., 1997; Koenig et al., 1998; Schwartkop et al., 1999; Elberg et al., 2002; Liu et al., 2003). Therefore, we first characterized the effects of somatostatin analogs on sst2 receptor internalization in two model systems, HEK293 and CHO-K1 cells, which have been used extensively for studies of receptor endocytosis (Menard et al., 1997). The cell lines were produced by clonal isolation after stable transfection. Saturation binding assays with [^{125}I]-[Tyr 3]octreotide gave a receptor density of 3.0 ± 0.2 pmol/mg CHO-sst2 membrane protein and 8.9 ± 0.5 pmol/mg HEK-sst2 membrane protein and K_d values of 0.093 ± 0.026 nM with CHO-sst2 membranes and 0.048 ± 0.026 nM with HEK-sst2 membranes.

The sst2 receptor was rapidly internalized after somatostatin stimulation in both HEK-sst2 cells and in CHO-sst2 cells (Fig. 1). In the absence of somatostatin, the receptor was localized primarily at the plasma membrane in both cell

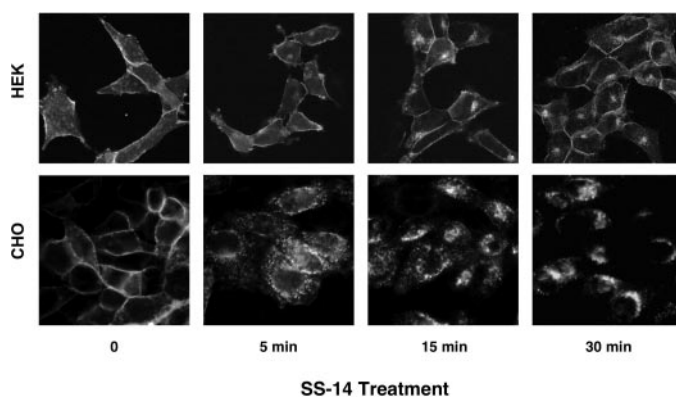


Fig. 1. Somatostatin induced internalization of the sst2 receptor in HEK293 and CHO-K1 cells. Top, HEK293 cells stably expressing the T7 epitope-tagged sst2A receptor (HEK-sst2 cells) were treated with 100 nM SS-14 for 0, 5, 15, and 30 min at 37°C. The cells were then fixed, permeabilized, and labeled with R2-88 antiserum and processed for immunocytochemistry as described under *Materials and Methods*. Bottom, CHO-K1 cells stably expressing the HA-tagged sst2A receptor (CHO-sst2 cells) were preincubated with anti-HA monoclonal antibody to label cell surface receptors. After removing unbound antibody, cells were treated with 100 nM SS-14 for 0, 5, 15, and 30 min at 37°C. The cells were then fixed, permeabilized, and processed for immunocytochemistry as described under *Materials and Methods*.

types. After 5 min of SS-14 exposure, punctate perinuclear staining was observed in HEK-sst2 cells in addition to the membranous staining, whereas in CHO-sst2 cells, the majority of the receptors were observed in cytoplasmic vesicles. By 15 min of SS-14 treatment, intracellular sst2 receptors were concentrated in the perinuclear region in both cell types, and their localization remained unchanged after 30 min. It is interesting that whereas cell surface receptors were undetectable in CHO-sst2 cells after 30 min of SS-14 exposure, in HEK-sst2 cells, a significant fraction of the receptors remained at the cell surface in the continued presence of SS-14. In summary, although the extent of receptor internalization finally achieved was somewhat different in the two cell types, in both cases, a new steady-state distribution of receptors was achieved within 15 min of SS-14 treatment.

sst2 receptor internalization is inhibited by hypertonic sucrose and thus occurs via clathrin-coated vesicles (Koenig et al., 1998; Hipkin et al., 2000). To identify the intracellular compartment containing the sst2 receptor after agonist induced internalization, we compared its distribution with that of the cation-independent M6PR, a TGN and late endosomal marker protein (Lin et al., 2004). Because of the specificity of the M6PR antibody, only cells of human origin could be used for this experiment. Agonist stimulation of HEK293 cells for 20 min caused translocation of the sst2 receptor from the plasma membrane to endocytic compartments containing M6PR (Fig. 2). Therefore, the internalized sst2 receptor is targeted to the TGN-late endosomal compartment.

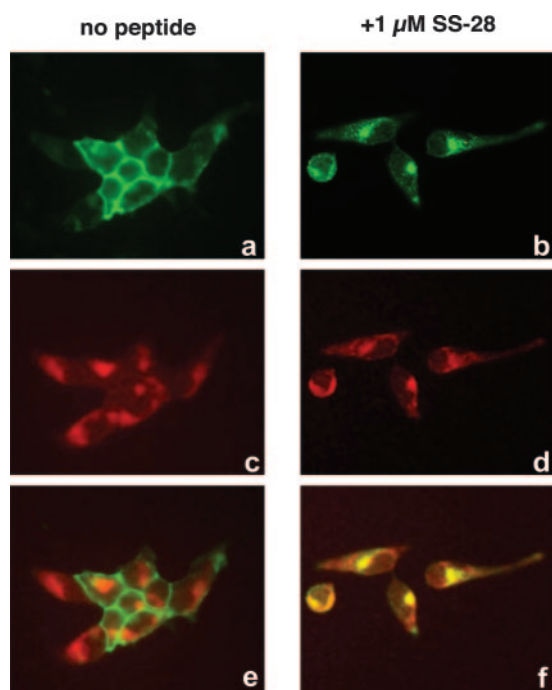


Fig. 2. Internalized sst2 colocalizes with the cation-independent M6PR, a TGN/late endosomal marker protein. HEK-sst2 cells were treated without (a, c, e) or with (b, d, f) 1 μ M SS-28 for 20 min at 37°C. The cells were then fixed, permeabilized, and incubated with sst2 antiserum (R2-88) and monoclonal M6PR-antibody. Alexa Fluor 488 goat anti-rabbit IgG (H+L) and Alexa Fluor 546 goat anti-mouse IgG (H+L) were used as secondary antibodies. The same cells are shown in a, c, and e and b, d, and f, with green labeling for the sst₂ receptor (a and b), red labeling for the M6PR (c and d). The merged images (e and f) demonstrate the absence of receptor colocalization (e) in control cells and the presence of receptor colocalization (f) in SS-28 treated cells.

Effect of sst2 Agonists and Antagonists on Receptor Internalization. We next determined the effect of several well characterized peptide (SS-14, cortistatin-17, vapreotide) and nonpeptide (L-779,976) agonists on sst2 receptor endocytosis (see Table 1 for structures). The results in Fig. 3 demonstrate that all the agonists induced receptor endocytosis in HEK293 cells. In contrast, sst2 internalization was not stimulated by the sst1 receptor-selective agonist CH-288 (data not shown). The order of potency for the compounds tested was: L-779,976 > SS-14 = vapreotide > cortistatin-17. In CHO-sst2 cells, saturating concentrations of both peptide (cortistatin-17, vapreotide) and nonpeptide (L-779,976) agonists also produced receptor internalization similar to that produced by SS-14 (compare Fig. 4 and Fig. 1, bottom).

In contrast to agonists, the sst2 antagonists Coy-14 (Rajeswaran et al., 2001) and CYN-154806 (Bass et al., 1996) did not induce receptor endocytosis in either CHO-sst2 cells (Fig. 4) or HEK-sst2 cells (data not shown). In fact, both compounds completely blocked agonist-induced internalization (Fig. 5), whereas a somatostatin analog that behaves as an antagonist at the sst3 receptor (sst3-ODN-8) was inactive (data not shown). Thus, full agonists stimulated sst2 receptor endocytosis in a dose-dependent manner whereas antagonists did not.

Quantitative Analysis of sst2 Receptor Endocytosis. Although immunocytochemical staining sensitively detects agonist induced receptor internalization and provides an indication of the rank order of different agonists in eliciting this response, it is not sufficiently quantitative to allow analog potencies and efficacies to be accurately determined. Therefore, we developed an ELISA assay to allow quantitation of receptor endocytosis.

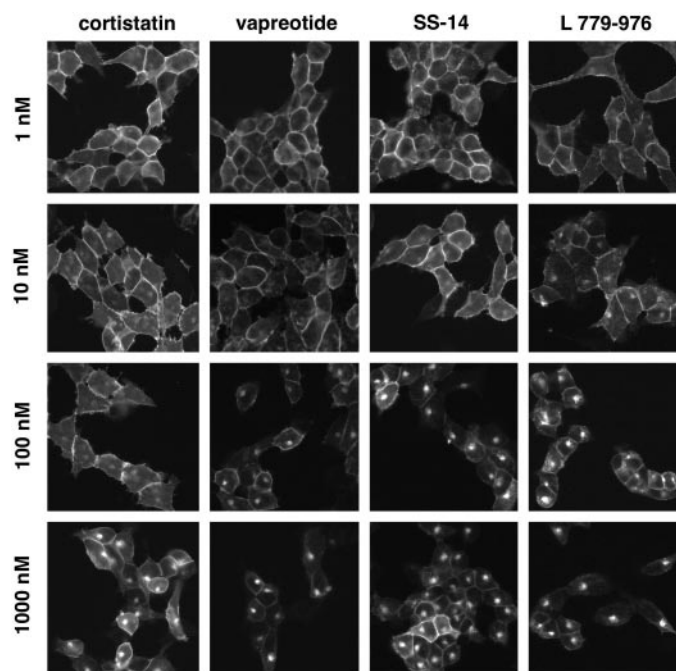


Fig. 3. Concentration dependence of agonist-induced receptor internalization in HEK293-sst2 cells. Cells were treated for 20 min at 37°C with 1, 10, 100, and 1000 nM concentrations of the agonists cortistatin-17, vapreotide, SS-14, and L-779,976. The cells were then fixed, permeabilized, labeled with sst2 antiserum, and processed for immunocytochemistry. A clear rank order of internalization potency is observed with L-779,976 > SS-14 = vapreotide > cortistatin-17.

Preliminary immunostaining experiments showed that pre-bound HA-antibody did not induce significant intracellular accumulation of sst2 receptor in the absence of ligand. To specifically measure ligand-stimulated endocytosis, we prelabeled cell surface receptors by incubating CHO-sst2 cells with HA antibody at room temperature. After washing to remove unbound antibody, cells were incubated for 30 min at 37°C, and ligand was added at various times before the end of the incubation to stimulate cells. All incubations were terminated simultaneously by chilling the cells to 4°C and then fixing with 3% paraformaldehyde before performing ELISA to quantitate receptor bound antibody remaining at the cell surface.

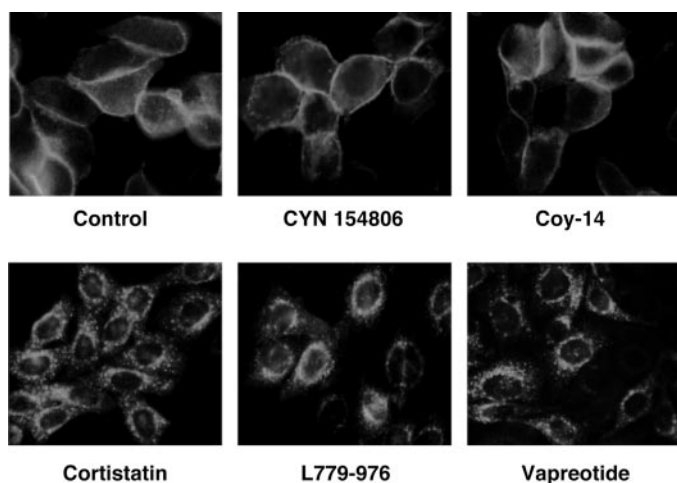


Fig. 4. Effects of agonists and antagonists on sst2 receptor internalization in CHO-sst2 cells. Cells were preincubated with HA monoclonal antibody and subsequently treated for 30 min at 37°C with either vehicle, or 100 nM concentrations of the antagonists CYN 154806 and Coy-14, or the agonists Cortistatin-17, L-779,976, and vapreotide. Cells were then fixed, permeabilized, incubated with the goat anti-mouse Cy-2 antibody and processed for immunocytochemistry.

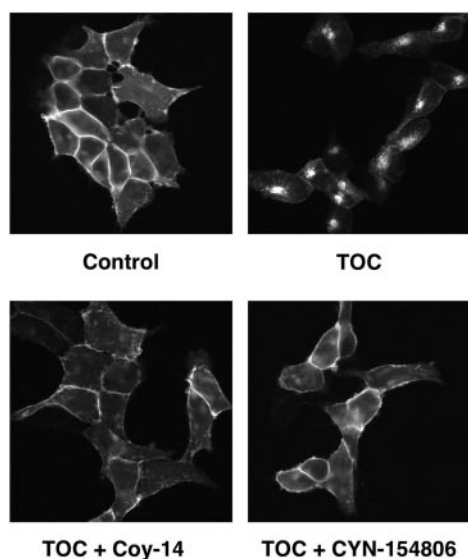


Fig. 5. Coy-14 and CYN-154806 prevent [Tyr³]octreotide-induced internalization of the sst₂ receptor. HEK-sst2 cells were treated for 20 min at 37°C with 100 nM [Tyr³]octreotide (TOC), 100 nM TOC plus 500 nM CYN-154806, or 100 nM TOC with 500 nM Coy-14. Untreated cells were used as control. After incubation with peptides, the cells were fixed, permeabilized, labeled with R2-88 antiserum, and processed for immunocytochemistry. In the same experiment, the sst3 receptor antagonist sst3-ODN-8 (500 nM) did not affect TOC-induced sst2 internalization (not shown).

Addition of a saturating concentration of the antagonists Coy-14 or CYN-154806 did not affect sst2A receptor endocytosis (Fig. 6). In contrast, the agonist octreotide (100 nM) caused rapid endocytosis of the sst2A receptor in CHO-sst2 cells, as we had observed by immunofluorescence microscopy. Fitting these data to a first-order rate equation gave the half-time of receptor endocytosis as 3.9 min. In multiple independent experiments, the half-times for receptor endocytosis were determined to be 3.2 ± 1.1 and 3.0 ± 0.7 min in the presence of 100 nM octreotide ($n = 2$) and 100 nM SS-14 ($n = 5$), respectively. Maximal concentrations of the analogs SS-28 (100 nM), vapreotide (100 nM), and KE108 (1 μ M) produced the same time course of receptor endocytosis as octreotide and SS-14 (data not shown).

We next determined the potencies of the different agonists to stimulate sst2 receptor internalization. Figure 7 shows typical results from two experiments and demonstrates that the data fit a single-site model. The EC₅₀ values for the sst2 receptor ligands tested in multiple experiments are summarized in Table 2. In general, the order of analog potencies determined in CHO-sst2 cells agreed with the more qualitative results obtained in HEK-sst2 cells using immunofluorescence microscopy (Fig. 3).

In CHO-sst2 cells, the agonists tested showed a 100-fold range of potencies and exhibited the same efficacy for receptor endocytosis as SS-14, with one exception: the nonpeptide analog L-779,976 was unable to induce maximal receptor internalization (Fig. 7). In three independent experiments, L-779,976 elicited 73% of the endocytosis produced by somatostatin (SS-14/SS-28) ($P = 0.017$ by paired t test). Moreover, neither CYN-154806 nor Coy-14 induced sst2 receptor endocytosis. In fact both compounds blocked SS-14-induced sst2 receptor internalization in a dose-dependent manner (Fig. 8).

Assessment of Agonist-Promoted β arrestin-GFP Translocation and Trafficking. To investigate the molecular basis for the observed difference in the efficacy of SS-14

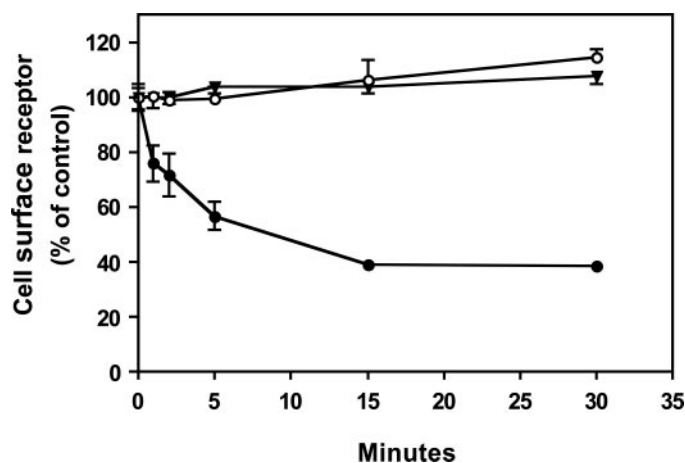
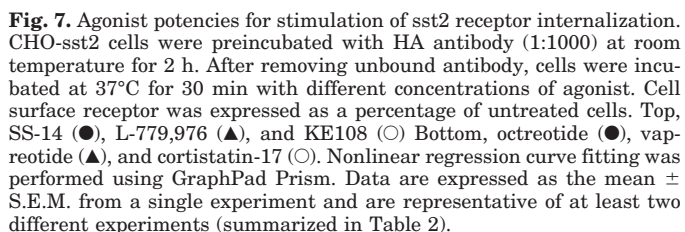


Fig. 6. Quantitation of ligand induced sst2 internalization by ELISA. CHO-sst2 cells were preincubated with HA antibody (1:1000) at room temperature for 2 h. After washing to remove unbound antibody, cells were incubated at 37°C for various times with 100 nM concentrations of octreotide (SMS 201-995; ●), CYN-154806 (▼), or Coy-14 (○). The cells were then fixed with paraformaldehyde and the receptors remaining at the cell surface were measured as described under *Materials and Methods*. Surface receptor at each time point was calculated as a percentage of the untreated control group. The half-time for octreotide-induced receptor internalization showed first-order kinetics and was determined to be 3.9 ± 0.1 min (GraphPad Prism).

monitor its association with the receptor during the endocytic process.

We quantitated the results of this experiment to ensure that the data shown were representative of all cells. After 5 min of SS-14 treatment, β arrestin-2-GFP was distributed in punctate, intracellular endosomal compartments in 59% of the cells (92 of 155). In the remainder of the cells, β arrestin-2-GFP was present in the cytosol. The latter group of cells expressed more fluorescent β arrestin-2 than the former, making it difficult to observe translocation of a portion of the expressed β arrestin 2. After 15 min of SS-14 treatment, β arrestin 2-GFP was localized in endocytic vesicles in 56% (132 of 238) of the cells. At both times, sst2 receptor was present in the same endocytic vesicles as β arrestin2-GFP. In contrast, after a 5-min treatment with L-779,976, only 0.7% of the cells (24 of 358) contained β arrestin 2-GFP in endosomal compartments; in 99.3% of the cells, β arrestin 2-GFP was uniformly distributed in the cytosol. After a 15-min treatment with L-779,976, 12.5% of the cells (42 of 335) contained vesicular β arrestin 2-GFP, and 87% of the cells showed uniform cytosolic distribution of β arrestin 2-GFP. In a second independent experiment, we again observed a dramatic difference in the rate of β arrestin 2-GFP dissociation



Analog binding affinities and potencies for stimulation of sst2 receptor endocytosis and signaling

Agonists are ordered according to their binding affinities (K_d values), which were determined in membrane binding assays as reported previously (see Reference column). Dose responses for agonist stimulation of receptor internalization and inhibition of forskolin-stimulated cAMP production were measured as described in Fig. 9. The EC_{50} values were calculated by nonlinear regression curve fitting as described under *Materials and Methods* and are given as mean \pm S.E.M. of the EC_{50} values obtained in the number of independent experiments shown in parentheses.

Analog	EC ₅₀			K _d Membrane Binding	Reference for K _d Value
	Receptor Internalization	CAMP Inhibition	Internalization/ cAMP		
		<i>nM</i>		<i>nM</i>	
L-779,976	0.56 ± 0.12 (3)	0.09 ± 0.01 (3)	6.2	0.05	Rohrer et al., 1998
SS-28	0.92 ± 0.07 (2)	1.42 ± 0.43 (4)	0.7	0.05	Nunn et al., 2004
SS-14	1.02 ± 0.11 (8)	0.73 ± 0.13 (14)	1.4	0.06	Nunn et al., 2004
Vapreotide	6.62 ± 0.47 (2)	0.75 ± 0.20 (4)	8.8	0.06	Nunn et al., 2004
[Tyr ³]Octreotide	0.8 ± 0.28 (2)	0.08 ± 0.02 (6)	10.0	0.12	Nunn et al., 2004
Octreotide	0.67 ± 0.12 (3)	0.16 ± 0.05 (4)	4.2	0.14	Nunn et al., 2004
KE108	54.5 ± 10.0 (4)	12.1 ± 3.3 (3)	4.5	0.90	Reubi et al., 2002
SOM230	25.9 ± 1.9 (2)	17.5 ± 5.1 (3)	1.5	1.00	Lewis et al., 2003
Cortistatin-17	11.3 ± 0.7 (2)	10.9 ± 2.7 (5)	1.0	1.43	Siehl et al., 1999

from the sst2 receptor when occupied by SS-14 and L-779,976.

The rapid release of β arrestin-2-GFP after endocytosis of the sst2 receptor–L-779,976 complex compared with its continuous tight association with intracellular sst2 receptors occupied by SS-14 suggests that the receptor conformations produced by these two analogs bind β arrestin with different affinities. Differential β arrestin binding to receptors occupied by different agonists is likely to account for variations in analog efficacies for inducing receptor endocytosis.

Comparison of Agonist Activities for Receptor Endocytosis and cAMP Inhibition. We next compared the potencies of agonists to induce sst2 receptor endocytosis with their previously reported binding affinities, as determined in radioligand displacement assays using membrane preparations in the absence of guanine nucleotides (Table 2). The data demonstrate that analogs were consistently less potent at inducing receptor endocytosis than in binding to the high-affinity form of the receptor. Moreover, the rank order for internalization and binding were strikingly different (Table 2). However, these two assays are carried out under very different conditions. Therefore, we next compared the potencies and efficacies of agonists on signal transduction and receptor internalization, assays performed in intact cells un-

der similar conditions. Typical dose-response curves to stimulate receptor endocytosis and to inhibit cAMP production are shown for SS-14 and [Tyr³]octreotide in Fig. 11. Results from multiple such experiments are summarized in Table 2.

All agonists tested, including L-779,976, produced the same maximal inhibition of cAMP formation in CHO-sst2 cells as SS-14 and but varied by more than 200-fold in their potencies to inhibit cAMP production. Neither CYN-154806 nor Coy-14 altered cAMP levels at concentrations up to 1 μ M, the highest dose tested (data not shown). It is interesting, however, that agonists differed greatly in their relative potencies to inhibit cyclic AMP and induce receptor endocytosis (Table 2). For example, whereas [Tyr³]octreotide was approximately 10 times more potent than SS-14 or SS-28 at inhibiting cyclic AMP production, the three peptides were equipotent at stimulating receptor endocytosis (Fig. 11 and Table 2). In general, the native peptides SS-14, SS-28, and Cortistatin-17 showed similar potencies for cAMP inhibition and receptor endocytosis, whereas short peptide analogs such as vapreotide, octreotide, [Tyr³]octreotide, and KE108 were substantially more potent at inhibiting cAMP synthesis than inducing receptor internalization (Table 2). The nonpeptide agonist L-779,976 was in the latter group; although it was one of the most potent inhibitors of cyclic AMP production, it was approximately six times less potent at inducing endocytosis. Moreover, as shown in Fig. 7, L-779,976 was unable to induce the same maximal endocytosis as other agonists.

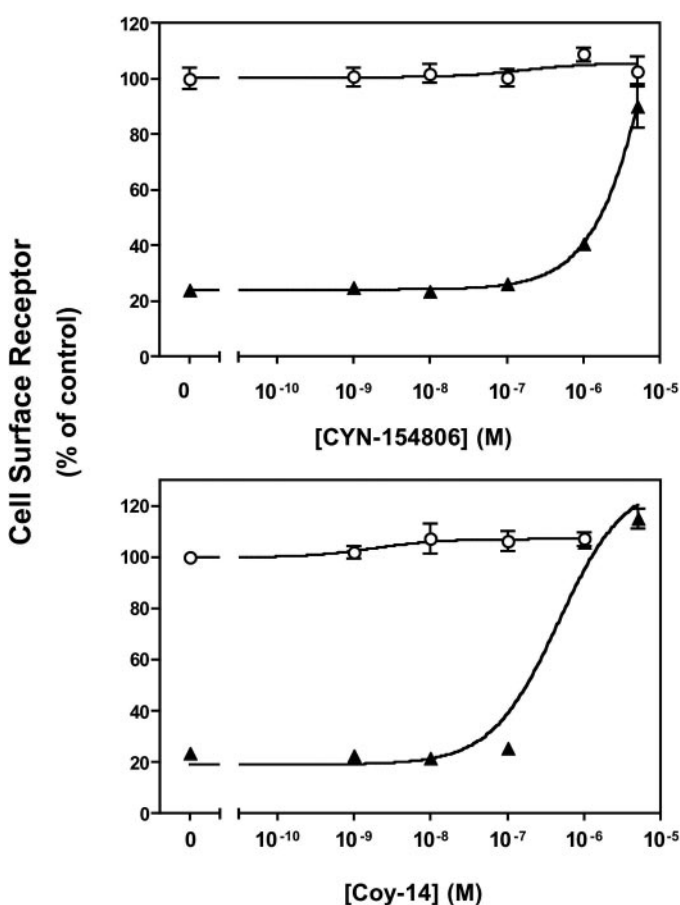


Fig. 8. Dose dependence for antagonist inhibition of sst2 receptor internalization. CHO-sst2 cells were preincubated with HA antibody at room temperature for 2 h. After removing unbound antibody, cells were incubated at 37°C for 30 min with different concentrations of CYN-154806 (top) or Coy-14 (bottom) either in the absence (○) or presence (▲) of 100 nM SS-14. Cell surface receptor was measured by ELISA and is expressed as a percentage of untreated cells.

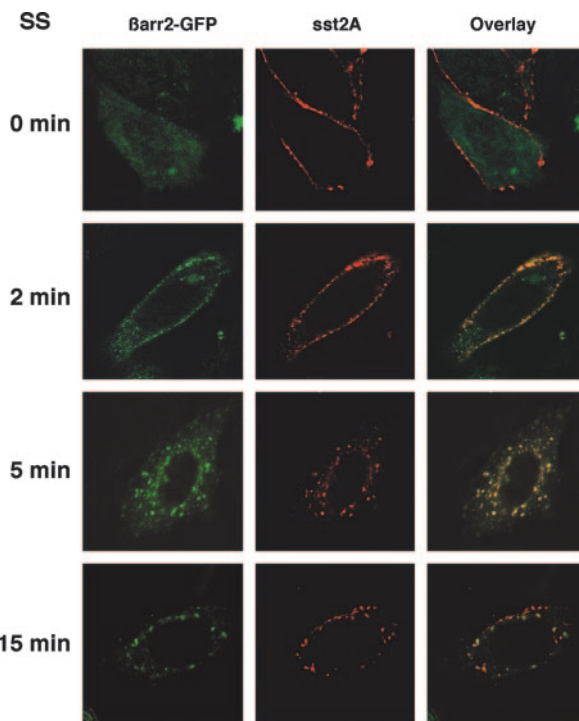


Fig. 9. Translocation and trafficking of β arrestin-2-GFP with the sst2 receptor after stimulation with SS-14. CHO-sst2 cells were transiently transfected with β arrestin2-GFP as described under *Materials and Methods*. After 24 h, cells were incubated with HA antibody on ice to label cell surface receptors. Cells were then treated with 100 nM SS-14 at 37°C for the times shown and subsequently fixed, permeabilized, and stained as described under *Materials and Methods*. Images were acquired with a Zeiss Confocal Laser Scanning 510 Microscope. The same cells are shown at each time point with green fluorescence showing the distribution of β arrestin-2-GFP and red fluorescence showing the distribution of the sst₂ receptor. The merged images show β arrestin-2 and receptor colocalization.

The marked differences in the signaling/endocytosis potency ratios for somatostatin analogs demonstrate that the effect of agonists to stimulate receptor internalization does not closely correlate with their effect on receptor signaling, just as it does not correlate with their binding affinities. These results provide compelling evidence for the induction of agonist-specific states of the sst2 receptor and show that no single measure of agonist activity is predictive of all post-receptor effects induced by agonists.

Discussion

We present the first quantitative comparison of the effect of different sst2 receptor agonists and antagonists on signaling and endocytosis, two therapeutically critical activities of this receptor. We show for the first time that two peptides reported to behave as antagonists, Coy-14 and CYN-154806, are unable to induce receptor endocytosis and block the effect of agonists to do so. Furthermore, sst2 agonists differ in their potency ratios for inhibiting adenylyl cyclase and stimulating receptor internalization. Our results provide the first evidence that ligand-specific receptor conformations occur for the sst2 somatostatin receptor and lay the groundwork for the design of analogs that target specific sst2 functions.

The regulation of GPCRs has been extensively investigated, and numerous common elements have been identified in the molecular mechanisms involved. Nonetheless, significant variation has been observed among different receptors.

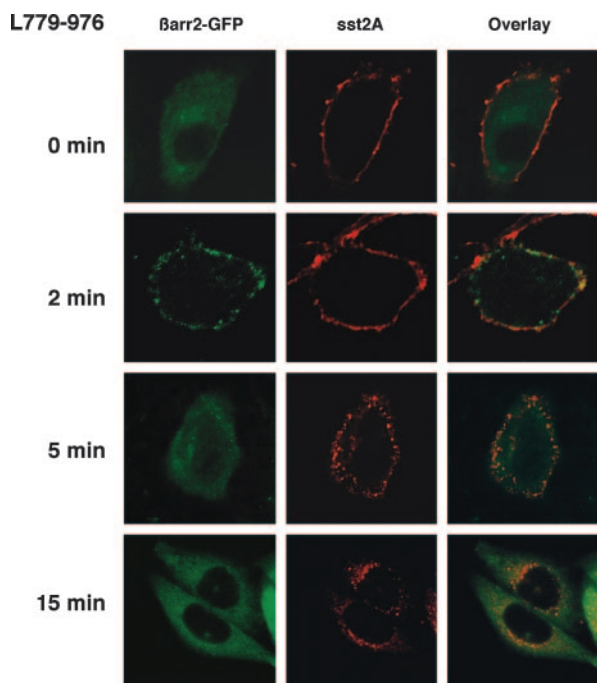


Fig. 10. Transient translocation of β arrestin-2-GFP to the sst2 receptor after stimulation with L-779,976. CHO-sst2 cells were transiently transfected with β arrestin2-GFP. After 24 h, cells were incubated with HA antibody on ice to label cell surface receptors. Cells were then treated with the nonpeptide somatostatin analog L-779,976 (100 nM) at 37°C for the times shown and subsequently fixed, permeabilized, and stained as described under *Materials and Methods*. Images were acquired with a Zeiss Confocal Laser Scanning 510 Microscope. The same cells are shown at each time point, with green fluorescence showing the distribution of β arrestin-2-GFP and red fluorescence showing the distribution of the sst₂ receptor. The merged images show β arrestin-2 and receptor colocalization.

The trafficking and regulation of sst2 somatostatin receptors has been of particular interest because of its importance for the detection and treatment of receptor-containing tumors by radioligand targeting as well as its impact on the responsiveness of tumors to somatostatin analog therapy (Breeman et al., 2001; Hofland and Lamberts, 2003; Reubi, 2003). However, detailed studies are lacking, and much of our current thinking is derived by extrapolation from observations made with other GPCRs (Csaba and Dournaud, 2001; Schonbrunn, 2001, 2004; Olias et al., 2004). Agonist binding has been shown to stimulate sst2A receptor phosphorylation, and this stimulation is potentiated by overexpression of GPCR kinases (GRKs) (Hipkin et al., 1997; Schwartkop et al., 1999; Tulipano et al., 2004). Receptor phosphorylation occurs within minutes of agonist treatment in both transfected cell

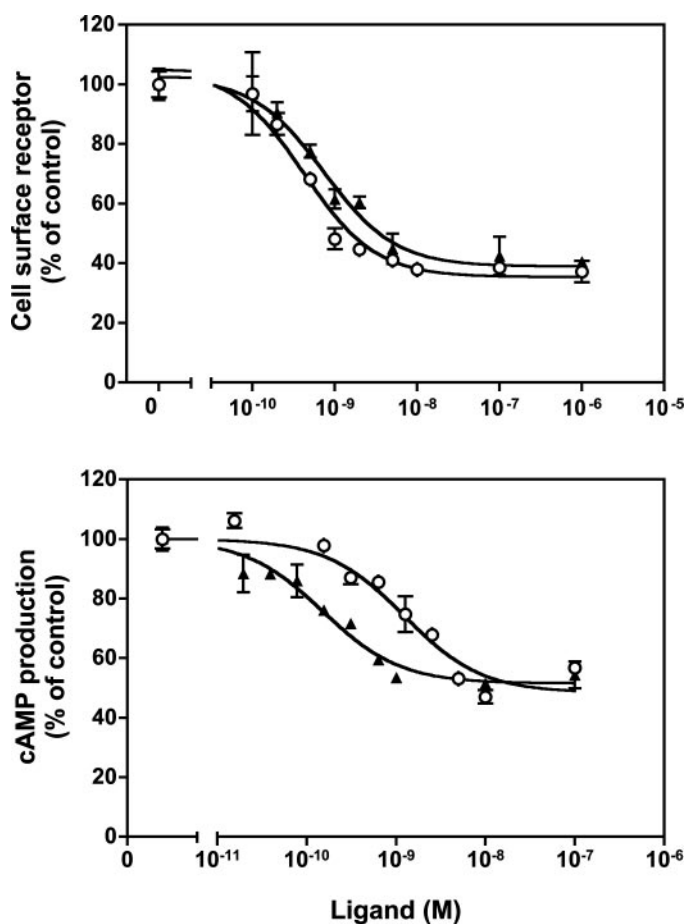


Fig. 11. Agonist potencies for stimulation of receptor internalization and inhibition of cAMP production. Top, CHO-sst2 cells were preincubated with HA antibody at room temperature for 2 h. After removing unbound antibody, cells were incubated at 37°C for 30 min with different concentrations of either SS-14 (○) or [Tyr³]octreotide (▲), and cell surface receptor was measured by ELISA as described under *Materials and Methods*. Surface receptor is expressed as a percentage of untreated cells. Bottom, triplicate wells of CHO-sst2 cells, preincubated with 3.5 μ Ci of [³H]adenine for 3 h at 37°C, were subsequently incubated with PDE inhibitor (1 mM IBMX) for 5 min. Cells were then treated at 37°C for 10 min with 10 μ M forskolin in the presence of different concentrations of either SS-14 (○) or [Tyr³]octreotide (▲). Reactions were stopped by the addition of 5% TCA and intracellular cAMP was measured as described in *Materials and Methods*. Cyclic AMP in each group is expressed as a percentage of that measured in cells treated with forskolin alone. Data are expressed as the mean \pm S.E.M. from a single experiment and are representative of multiple different experiments (summarized in Table 2).

lines (Hipkin et al., 1997; Schwartkop et al., 1999; Tulipano et al., 2004) and cells expressing the receptor endogenously (Elberg et al., 2002). Moreover, agonist-stimulated receptor phosphorylation is correlated with receptor internalization, not only in cultured cells (Hipkin et al., 1997) but also in human tumors in vivo (Liu et al., 2003), suggesting that the two processes are causally linked. However, a T7-tagged sst2A receptor construct lacking the last 44 amino acids of the C terminus was shown to undergo agonist-induced internalization in the absence of detectable receptor phosphorylation in HEK293 cells (Schwartkop et al., 1999). Thus, although the sst2A receptor is subject to agonist-stimulated phosphorylation by GRKs, the necessity of GRK-mediated receptor phosphorylation for its endocytosis remains to be demonstrated conclusively.

SS-14 binding also stimulates β arrestin recruitment to the sst2A receptor at the plasma membrane (Brasselet et al., 2002; Tulipano et al., 2004). Although β arrestin is endocytosed with the receptor after SS-14 stimulation (Brasselet et al., 2002; Tulipano et al., 2004), it was proposed to play a role in desensitization rather than receptor internalization, because expression of a dominant-negative β arrestin failed to inhibit sst2 endocytosis (Brasselet et al., 2002). Thus, although both GRKs and β arrestins have been implicated in sst2A regulation, their precise functions remain unknown.

The cellular concentrations of β arrestins and GRKs can have marked effects on the rate of endocytosis of GPCRs (Menard et al., 1997). To select an optimal system to measure the effects of analogs on sst2A receptor endocytosis, we compared the trafficking of this receptor in CHO-K1 and HEK293 cells using immunofluorescence microscopy. These two cell lines have been used extensively for studies of receptor internalization and have been reported to contain a comparable complement of GRKs and β arrestins, even though the levels of specific GRKs and β arrestins differ (Menard et al., 1997).

We observed similar rates of sst2A receptor endocytosis in HEK-sst2 and CHO-sst2 cells at saturating ligand concentrations, although the extent of receptor internalization observed at steady state was somewhat greater in CHO cells. In both cell types, 0.45 M sucrose blocked sst2A endocytosis (data not shown), confirming previous results (Koenig et al., 1998; Hipkin et al., 2000) and demonstrating that receptor internalization occurs via clathrin-coated vesicles. Furthermore, in both cell lines, the sst2A receptor trafficked to endocytic compartments that were identified in HEK-sst2 cells as the late endosomal compartment containing the mannose 6-phosphate receptor. Finally, somatostatin analogs behaved similarly in the two cell lines, both with respect to the rank order of agonist potencies for stimulation of endocytosis and in the inability of antagonists to induce receptor endocytosis. It is interesting, however, that we observed subtle differences in the pattern of intracellular vesicles with which the receptor was associated early in the endocytic process. The biochemical significance of this difference remains to be determined. We selected the CHO-sst2 cells for further quantitative analysis to take advantage of the greater extent of internalization at steady state.

Quantitation of sst2 endocytosis by ELISA provides several advantages over previous studies using radiolabeled ligands. The assay directly measures the internalization of the receptor itself rather than the bound ligand. Thus, the

ELISA is not limited by the ability to radiolabel the analogs to be tested and is not affected by changes in analog structure produced by the introduction of radioactive iodine. Furthermore, receptor internalization can be quantitated at a large range of agonist concentration and receptor occupancy, rather than only at subsaturating radioligand concentrations. The ELISA is also preferable to assays that measure cell surface receptors by radioligand binding after dissociation of the unlabeled agonists used to drive receptor endocytosis. Such post-treatment radioligand binding assays require incubating cells with acid to dissociate prebound ligand, a procedure that may not fully and equally dissociate all high-affinity receptor-ligand complexes. The ELISA assay avoids not only problems produced by incomplete dissociation of prebound ligand but also any denaturation of receptor by the acid treatment used for ligand release.

Having validated our ELISA by showing that the assay results paralleled the data obtained using the qualitative immunofluorescence assay, we used it to analyze receptor internalization in the presence of a series of well characterized sst2 receptor agonists. Previous studies with opioid receptors showed that nonpeptide agonists such as morphine were less effective inducers of receptor endocytosis than peptide agonists (Borgland et al., 2003; Li et al., 2003; von Zastrow et al., 2003). Because somatostatin and opioid receptors share substantial sequence similarity (Schonbrunn, 2001, 2004), comparison of the efficacy of the nonpeptide agonist L-779,976 with that of native somatostatin and its peptide analogs was of particular interest. Previous studies showed that L-779,976 bound to sst2 receptors with the same affinity as the native ligands SS-14 and SS-28 (Rohrer et al., 1998). However, L-779,976 was approximately 10 times more potent than SS-14 and SS-28 for inducing cyclic AMP inhibition, whereas it was only twice as potent for stimulating receptor endocytosis. Moreover, L-779,976 was less efficacious than the agonist peptides at stimulating receptor internalization; at maximal doses, L-779,976 produced 73% the effect of SS-14 (Fig. 7). This differential effect suggests that nonpeptide sst2A analogs might be designed to allow complete dissociation of these two consequences of receptor activation.

Previous studies demonstrated that treatment with SS-14 leads to the binding of β arrestin-1-GFP and β arrestin-2-GFP to cell surface sst2 receptors and that the receptors remain complexed with β arrestins after internalization (Brasselet et al., 2002; Tulipano et al., 2004). Our data confirmed these results. In addition, we observed a different interaction between sst2 and β arrestin-2-GFP after treatment with the nonpeptide agonist L-779,976. Although β arrestin-2-GFP was also recruited to plasma membrane sst2 receptors after L-779,976 binding, the β arrestin-receptor complex dissociated within a few minutes. This observation indicates that the SS-14-receptor complex binds β arrestin with higher affinity than L-779,976-receptor complex. Therefore, it seems likely that the lower efficacy of L-779,976 compared with SS-14 for stimulating sst2 receptor endocytosis results from the lower affinity of β arrestin for the receptor conformation induced by this ligand. Additional studies will be required to determine whether this difference in receptor conformation is directly responsible for the observed decrease in β arrestin affinity or affects it indirectly, perhaps as a result of differences in receptor phosphorylation by GRKs.

Comparison of the rank order of potencies for a number of peptide analogs provided additional evidence that inhibition of adenylyl cyclase and receptor internalization could be differentially regulated. The endogenous ligands, SS-14, SS-28, and cortistatin all exhibited approximately equal potencies for inducing these two consequences of sst2A receptor activation. In contrast, many of the small, stable peptide analogs either already in clinical use, such as octreotide, or being investigated for therapeutic applications, such as vapreotide and KE108, were 5 to 10 times more potent at signaling to cyclase than for stimulating receptor endocytosis. This difference in the relative potencies of agonists demonstrates that receptor activation and internalization are not tightly linked. Furthermore, our data provide the first evidence for the existence of distinct ligand-induced conformations for the sst2A receptor, conformations that have differential effects on Gi/o activation and β arrestin binding (Kenakin, 2003).

The effect of antagonists on sst2A receptor endocytosis has not previously been examined. Although antagonists do not stimulate the internalization of most GPCRs, there have been notable exceptions (Roettger et al., 1997; Bhowmick et al., 1998; Gray and Roth, 2001; Pheng et al., 2003). We examined two compounds, both of which have been reported to behave as sst2 antagonists. Confirming previous results (Rajeswaran et al., 2001), Coy-14 did not inhibit cAMP production in our experiments (data not shown). Furthermore, it did not induce sst2A receptor internalization and instead blocked agonist stimulation (Figs. 5 and 8). CYN-154806 was originally described as an sst2 receptor antagonist (Bass et al., 1996) but was subsequently shown to have partial agonist activity (Nunn et al., 2003). In our CHO-sst2 cells, CYN-154806 behaved as a pure antagonist for both receptor endocytosis (Figs. 5 and 8) and cyclic AMP regulation (data not shown).

In conclusion, we demonstrate that the activity of ligands to stimulate sst2 receptor signaling and endocytosis varies widely, providing the first evidence for distinct agonist-induced receptor conformations preferentially coupled to different functional pathways. These results indicate that analogs selective for different sst2 activities may provide a new approach for therapeutic targeting of sst2 receptor expressing tumors.

Acknowledgments

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