Differential β-Arrestin Trafficking and Endosomal Sorting of Somatostatin Receptor Subtypes*

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The physiological responses of somatostatin are mediated by five different G protein-coupled receptors. Although agonist-induced endocytosis of the various somatostatin receptor subtypes (sst1–sst5) has been studied in detail, little is known about their postendocytic trafficking. Here we show that somatostatin receptors profoundly differ in patterns of β-arrestin mobilization and endosomal sorting. The β-arrestin-dependent trafficking of the sst2A somatostatin receptor resembled that of a class B receptor in that upon receptor activation, β-arrestin and the receptor formed stable complexes and internalized together into the same endocytic vesicles. This pattern was dependent on GRK2 (G protein-coupled receptor kinase 2)-mediated phosphorylation of a cluster of phosphate acceptor sites within the cytoplasmic tail of the sst2A receptor. Unlike other class B receptors, however, the sst2A receptor was rapidly resensitized and recycled to the plasma membrane. The β-arrestin mobilization of the sst2 and the sst5 somatostatin receptors resembled that of a class A receptor in that upon receptor activation, β-arrestin and the receptor formed relatively unstable complexes that dissociated at or near the plasma membrane. Consequently, β-arrestin was excluded from sst5-containing vesicles. Unlike other class A receptors, a large proportion of sst2 receptors was subject to ubiquitin-dependent lysosomal degradation and did not rapidly recycle to the plasma membrane. The sst1 somatostatin receptor is unique in that it did not exhibit agonist-dependent receptor phosphorylation and β-arrestin recruitment. Together, these findings may provide important clues about the regulation of receptor responsiveness during long-term administration of somatostatin analogs.

Somatostatin (SS-14)† is an important regulator of neurotransmission in the brain as well as of hormone secretion from the anterior pituitary gland, the pancreas, and the gastrointestinal tract. Five genes encoding six different somatostatin receptor subtypes (sst1–sst6) have been cloned. These receptors are widely expressed in the central nervous system and periphery, and multiple somatostatin receptor subtypes often coexist in the same cell (1, 2). The high density of somatostatin receptors on human neuroendocrine tumors (3–5) has been used clinically to treat the symptoms of hormonal hypersecretion in patients with growth hormone- and thyrotropin-secreting pituitary adenomas and patients harboring islet cell or carcinoid tumors (6–8). Moreover, it has allowed the development of somatostatin receptor scintigraphy for tumor imaging as well as somatostatin receptor-targeted radiotherapy (9, 10). The fact that naturally occurring somatostatin peptides have only short half-lives has necessitated the development of stable somatostatin peptide analogs including octreotide and lanreotide. Although SS-14 binds with high affinity to all five somatostatin receptors, octreotide and lanreotide bind only to sst2 with high affinity and to sst3 and sst5 with moderate affinity (1, 11).

It is well known that the physiological responses to SS-14 are diminished with continued exposure (1). However, important differences have been observed in the response of neuroendocrine tumors to the long-term application of stable somatostatin analogs. In patients harboring somatostatin receptor-expressing growth hormone-secreting adenoma, the inhibitory effects of somatostatin analogs on hormone secretion persist for many years during long-term treatment, and only few thyrotropin-secreting tumors escape from octreotide therapy. In contrast, islet cell tumors and carcinoids are very likely to undergo desensitization within weeks to months of octreotide exposure (12). Although studies in transfected host cells have examined agonist-induced internalization of the various somatostatin receptor subtypes (2), the molecular basis for the distinct long-term responsiveness of individual target cells has not been established. So far only species-related differences in agonist-mediated endocytosis of somatostatin receptors have been observed, e.g. the rat sst1 receptor appears to be largely resistant to agonist-induced internalization and desensitization (13), whereas the human sst1 receptor has been reported to undergo a very slow but clearly detectable endocytosis. Other studies have shown that the human sst1 receptor but not the rat sst1 receptor failed to internalize in an agonist-dependent manner (14, 15).

The aim of the present study was to characterize early molecular events after agonist activation that lead to functional desensitization and sequestration of the distinct somatostatin receptor subtypes. Moreover, we wanted to delineate the molecular determinants that dictate the differential trafficking and endosomal sorting of somatostatin receptors. All five somatostatin receptors belong to the superfamily of G protein-
coupled receptors (GPCRs). After agonist binding and activation of GPCRs, the signaling is turned off by phosphorylation of intracellular receptor domains and subsequent recruitment of cytoplasmic proteins termed arrestins that interrupt coupling between the receptor and its cognate heterotrimeric G protein (16, 17). β-Arrtine also function as docking proteins that link the receptor to components of the endocytic machinery such as AP-2 and clathrin and as scaffolding proteins to turn on signaling to mitogen-activated protein kinase (MAPK) cascades (18). Within the endosomal compartment, β-arrestins regulate the rate at which internalized receptors are dephosphorylated and recycled to the plasma membrane (19, 20). Based on their binding properties to different isoforms of β-arrestin, GPCRs have been categorized into two classes. Class A receptors (e.g., μ-opioid, β2 and α1B adrenergic, endothelin A, and dopamine D1A receptors) do not bind visual arrestin and have a higher affinity for nonvisual β-arrestin-2 than β-arrestin-1. Class B receptors (e.g., substance P, angiotensin AT1a, neurotensin 1, and vasopressin 2 receptors) bind visual arrestin and have similar affinities for β-arrestin-1 and β-arrestin-2 (16). Class A and class B receptors also differ in the fate of the β-arrestin-receptor complex. For class A receptors, β-arrestin directs the receptors to clathrin-coated pits but does not internalize with them. For class B receptors, β-arrestin forms stable complexes with the receptors, such that the receptor-β-arrestin complex internalizes as an unit into early endosomes (17).

Here we identify the sst2 and sst3 somatostatin receptors as class A receptors and the sst2A somatostatin receptor as class B receptor. The trafficking of the sst2 and sst3 somatostatin receptors did not appear to depend on β-arrestins. We demonstrate that the formation of stable complexes between β-arrestin and sst3 requires G protein-coupled receptor kinase 2 (GRK2)-mediated phosphorylation of a cluster of phosphate acceptor sites within the cytoplasmic tail of the sst3 receptor. We also show that sst3 and sst2 underwent differential endosomal sorting. In contrast to what would be expected from their β-arrestin mobilization patterns, the sst2A receptor was rapidly recycled to the plasma membrane whereas the sst3 receptor was subject to ubiquitination-dependent lysosomal degradation.

EXPERIMENTAL PROCEDURES

Materials—Monoclonal mouse anti-ubiquitin antibody (UbP4D1) was from Santa Cruz Biotechnology. Mouse monoclonal anti-T7 antibody and mouse monoclonal anti-T7 antibody covalently coupled to Sepharose beads were obtained from Novagen (Madison, WI). The rabbit anti-sst2A antibody was generated to the peptide ETQRTLLNG-DLQTSE, which corresponds to residues 417–428 of the carboxy-terminal tail of the rat/mouse/human sst2A, which contains residues 417–428 of the carboxy-terminal tail of the rat/mouse sst2. Both antibodies have been characterized extensively (21). All polyclonal rabbit antisera were purified by affinity chromatography using the Sulfo-Light coupling gel coupled to the appropriate immunizing peptide according to the instructions of the manufacturer (Pierce). Plasmid constructs of the rat sst1, sst3A, sst2, and sst3, containing the amino-terminal T7 epitope tag sequence MASMGGQGMG in pcDNA3 have been described previously (13, 15, 21). Truncated sst2A mutants were created by PCR using primers that introduce stop codons at suitable positions (22). Point mutations were introduced into sst2A by PCR replacing threonine 353, 355, and 356 with alanine. The sequences of all constructs were verified by dideoxynucleotide sequencing. The plasmid encoding β-arrestin-1-enhanced green fluorescence protein (EGFP) was kindly provided by N. W. Bunning (University of California, San Francisco). The construct encoding the β-arrestin-2-EGFP fusion protein was obtained from Biosignal (Montreal, Canada). Transferrin-Alexa Fluor 488 was purchased from Molecular Probes (Leiden, The Netherlands). LipofectAMINE 2000 according to the instructions of the manufacturer (Invitrogen). Stable transfectants were selected in the presence of 500 μg/ml G418 (Invitrogen). HEK 293 cells stably expressing the T7-tagged sst2A (Bmax 820 ± 19 fmol/mg membrane protein) or T7-tagged sst2A receptors (Bmax 1,182 ± 31 fmol/mg membrane protein) were characterized using radioligand binding assays, Western blot analysis, and immunocytochemistry as described previously (21). The level of somatostatin receptor expression was between 1,500 and 2,000 fmol/mg membrane protein for all experiments using transiently transfected HEK 293 cells.

Confocal Microscopy—One day after transfection, cells were seeded into 35-mm glass-bottom culture dishes (Mattek, Ashland, MA). The next day, cells were incubated for 2 h in serum-free OPTIMEM-1 medium (Invitrogen) containing 10 mM Hepes (pH 7.4). Cells were then washed once onto a temperature-controlled microscope stage incubated at 37 °C. Confocal microscopy was performed using a Leica TCS NT laser scanning confocal microscope (Heidelberg, Germany). Analysis of β-arrestin translocation was performed in cells transiently cotransfected with 1.5 μg of either β-arrestin-1-EGFP or β-arrestin-2-EGFP and 6 μg of the various somatostatin receptor subtypes. When indicated, 4 μg of GRK2 or 4 μg of empty vector (MOCK) were included in the transfections. Images were collected sequentially using single line excitation at 488 nm with 515–540 nm band pass emission filters. Saturating concentrations of SS-14 (1 μM) were applied directly into the culture medium immediately after the initial image was taken.

Immunocytochemistry—Cells were grown on poly-l-lysine-coated coverslips overnight. After appropriate treatment with SS-14, cells were fixed with 4% paraformaldehyde and 0.2% picric acid in phosphate buffer (pH 6.9) for 40 min at room temperature and washed several times in TPBS (10 mM Tris-HCl, pH 7.4, 10 mM phosphate buffer, 137 mM NaCl, and 0.05% thimerosal). Specimens were then incubated for 3 min in 50% methanol and 3 min in 100% methanol, washed in TPBS, and preincubated with TPBS supplemented with 3% normal goat serum for 1 h at room temperature. Cells were then incubated with affinity-purified anti-T7, anti-sst2A, or anti-sst3 antibodies at a concentration of 1 μg/ml in TPBS supplemented with 1% normal goat serum overnight. After washing with TPBS, bound primary antibody was detected with cyanine 3.18-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA). Cells were then dehydrated, desorbed in xylol, and permanently mounted in DPX (Fluka, Deisenhofen, Germany). For β-arrestin-2-EGFP/sst2A colocalization studies, cells were mounted directly in Vectashield Hard Set mounting medium (Vector, Burlingame, CA). Specimens were examined using a Leica TCS NT laser scanning microscope. Cyanine 3.18 was imaged with 568 nm excitation and 570–630 nm band pass emission filters.

Transferrin Trafficking—A previously described pulse-chase assay was used to estimate the degree to which a “pulse” of internalized sst2A or sst3 was accessible to a subsequent “chase” of endocytosed transferrin (23). HEK 293 cells stably expressing T7-tagged sst2A or sst3 were grown on glass coverslips, and receptors were surface-labeled with 1 μg/ml 125I-transferrin. In the first set of experiments, cells were continuously incubated with 1 μM SS-14 and 5 μg/ml Alexa Fluor 488-conjugated transferrin for 30 min at 37 °C. In a second set of experiments, cells were first incubated with 1 μM SS-14 for 30 min at 37 °C to drive endocytosis of antibody-labeled receptors. Next, cells were chilled on ice, rinsed with EDTA-supplemented phosphate-buffered saline, rewarmed for 20 min in medium lacking agonist but containing 5 μg/ml Alexa Fluor 488-conjugated transferrin. Cells were fixed and permeabilized, and antibody-labeled sst2A or sst3 receptors were detected using cyanine 3.18-conjugated secondary antibodies. Specimens were examined as described above.

Whole Cell Phosphorylation Assay—HEK 293 cells were plated at a density of 105 cells/dish on 100-mm dishes. The next day, cells were transfected with the appropriate plasmids. Two days later, cells were washed with serum- and phosphate-free medium and then labeled with 200 μCi/ml carrier-free 32P orthophosphate (285 Ci/mg P, ICN, Eschwege, Germany) for 60 min at 37 °C. Labeled cells were then exposed to 1 μM SS-14 for 20 min. Subsequently, cells were placed on ice and washed with ice-cold phosphate-buffered saline. Cells were scraped into 1 ml of radioactive immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 10 mM disodium pyrophosphate, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.2% phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, and 10 μM bacitracin). Cells were solubilized on a rotating plate for 40 min at 4 °C. After centrifugation at 30,000 × g for 60 min at 4 °C, receptor proteins were immunoprecipitated using T7 affinity beads. Receptors were eluted from the beads using SDS-sample buffer for 20 min at 60 °C. The samples were subjected to 8% SDS-PAGE followed by autoradiography. The extent of
receptor phosphorylation was quantitated using a Fuji phosphoimaging system and BAS 1000 software. Loading of equal amounts of receptor proteins in each lane was confirmed by Western blot analysis. Receptor phosphorylation was expressed as a percent of wild-type sst2A. Means ± S.E. of three independent experiments are reported.

Western Blot Analysis—Stably transfected HEK 293 cells were plated onto poly-L-lysine-coated 100-mm dishes and grown to 80% confluence. After treatment with SS-14 in serum-free medium, cells were lysed in radioimmune precipitation buffer as described above. Glycosylated proteins were partially enriched using wheat germ-lectin-agarose beads (Amersham Biosciences). Proteins were eluted from the beads using SDS-sample buffer for 20 min at 60°C and then resolved on 8% SDS-polyacrylamide gels. After electroblotting, the membranes were incubated with 1 μg/ml anti-sst2A or anti-sst3 antibody for 12 h at 4°C followed by detection using an enhanced chemiluminescence detection system (Amersham Biosciences). For ubiquitination studies, 200 μM chloroquine (Sigma) and 25 μM MG132 (Calbiochem) were added to the culture medium 30 min before and during agonist treatment. Cells were then lysed in radioimmune precipitation buffer containing 200 μM chloroquine, 25 μM MG132, and 10 mM N-ethylmaleimide (Calbiochem). Receptor proteins were precipitated with T7 affinity beads. Ubiquitinated receptors were detected using anti-ubiquitin antibodies (2 μg/ml). NIH Image 1.62 software was used to desensitize and quantify protein bands detected on Western blots. Statistical analysis was carried out with the Student’s t test using GraphPad Prism 3.0 software. p-values of <0.05 were considered statistically significant.

RESULTS

Differential β-Arrestin Mobilization of Somatostatin Receptors—We employed functional β-arrestin-1 and β-arrestin-2 conjugated to enhanced green fluorescent protein to visualize the translocation of β-arrestins to the plasma membrane in live HEK 293 cells transfected with distinct somatostatin receptor subtypes. In the absence of the agonist, both β-arrestin-EGFP isoforms were uniformly distributed throughout the cytoplasm of the cells (Fig. 1, 0 min). β-Arrestin-1 was also detectable inside the cell nucleus. The addition of saturating concentrations of SS-14 induced a rapid redistribution of β-arrestin-1 as well as β-arrestin-2 from the cytoplasm to the plasma membrane in sst2A- and sst3-transfected cells. In sst3-expressing cells, fluorescence outlining the shape of the cells was less...
its trafficking with the receptor into the endocytotic vesicles (Fig. 3B, upper panel), indicating that these processes strongly depend on the cellular complement of GRK2. In sst₄-expressing cells, agonist-induced receptor phosphorylation (Fig. 3C, middle panel) and β-arrestin-2 trafficking (Fig. 3B, lower panel) were largely independent of the presence of overexpressed GRK2. Although the sst₄ receptor contains several putative phosphorylation sites in its cytoplasmic tail (Fig. 3A), we were not able to detect any agonist-induced receptor phosphorylation (Fig. 3C, right panel) or β-arrestin-2 mobilization (not shown) in the presence or absence of GRK2.

Molecular Determinants Underlying the Cotrafficking of β-Arrestin and sst2A into Endocytotic Vesicles—To determine the sites required for GRK2-mediated phosphorylation and β-arrestin mobilization of the sst₂A receptor, serial truncations were constructed deleting either 10 (356X), 15 (354X), or 20 (349X) amino acids of its carboxyl-terminal tail (Fig. 4A). As depicted in Fig. 4, B and C, deletion of 10 amino acids of the sst₂A receptor tail (359X mutant), which contain serine-368 and threonine-387 as potential phosphorylation sites, reduced GRK2-mediated phosphorylation by ~10% (residual phosphorylation, 90% ± 4%; n = 3) (Fig. 4C). Analysis of β-arrestin mobilization of the 359X mutant revealed that the receptor is still phosphorylated sufficiently enough to induce a translocation of β-arrestin-2-EGFP to the plasma membrane (Fig. 4B). However, analysis of the 359X receptor also showed that phosphorylation of serine 368 and/or threonine 367 as potential phosphorylation sites, reduced GRK2-mediated phosphorylation by ~40% (residual phosphorylation, 62% ± 14%; n = 3) (Fig. 4C). Analysis of β-arrestin trafficking of the 356X mutant revealed that the residual receptor phosphorylation was not sufficient to induce translocation of β-arrestin-2-EGFP to the plasma membrane (Fig. 4B). Deletion of 20 amino acids of the sst₂A receptor tail (349X mutant), which contain two additional potential phosphorylation sites, namely threonine 359 and threonine 356, reduced GRK2-mediated phosphorylation by ~62% (residual phosphorylation, 38% ± 3%; n = 3) (Fig. 4C). Analysis of β-arrestin trafficking of the 349X mutant revealed that the 356X mutant is targeted to the plasma membrane (Fig. 4B). Deletion of 20 amino acids of the sst₂A receptor tail (349X mutant), which contain two additional potential phosphorylation sites, namely threonine 359 and threonine 356, strongly reduced GRK2-mediated phosphorylation (residual phosphorylation, 19% ± 4%; n = 3) (Fig. 4C). In the AAEA mutant, the cluster of threonine 353, 355, and 356 was replaced by alanine residues. The level of agonist-induced phosphorylation was reduced by 55% as compared with the wild-type sst₂A (residual phosphorylation, 44% ± 2%; n = 3) (Fig. 4C). In cells expressing the AAEA mutant, agonist-induced β-arrestin-2 translocation was strongly inhibited, and fluorescence outlining the cell shape was hardly detectable. Immunocytochemical staining of nonpermeabilized cells using anti-T7 antibodies revealed that all sst₂A mutants were targeted to the plasma membrane (not shown). These data suggest that many if not all of the potential phosphate acceptor sites contained within the carboxyl-terminal amino acids are involved in GRK2-mediated phosphorylation of the sst₂A receptor and that phosphorylation of these sites is required for formation of stable β-arrestin-sst₂A complexes. These data also suggest that the cluster of threonine 353, 355, and 356 may represent the primary site for GRK2-mediated phosphorylation and β-arrestin binding.

Fate of sst₂A and sst₄ after Agonist-induced Internalization—The pattern of β-arrestin trafficking is believed to dictate the rate of receptor recycling and resensitization. In fact, many class A receptors have been shown to recycle rapidly, whereas class B receptor-like trafficking patterns are often observed for slowly recycling receptors. We therefore examined the redistribution of sst₂A and sst₄ after agonist-induced internalization. Cells were treated for 30 min with SS-14, washed extensively,
and subjected to an additional incubation in the absence of agonist for 10, 20, or 40 min. As depicted in Fig. 5, sst2A and sst3 were almost exclusively confined to the plasma membrane in untreated cells. After 30 min of SS-14 exposure, both receptors were extensively sequestered into intracellular vesicles. In the absence of agonist it became apparent that nearly the entire pool of internalized sst2A receptors recycled to the plasma membrane during the 40-min SS-14-free interval. In

![Fig. 3. Agonist-induced receptor phosphorylation and β-arrestin mobilization.](image)

A. carboxyl-terminal amino acid sequences of sst2A, sst3, and sst4 are depicted beginning with the conserved NPILY motif, which marks the end of the seventh transmembrane domain and the beginning of the cytoplasmic tail. Potential phosphate acceptor sites are underlined. B. HEK 293 cells were transiently transfected with 1.5 μg of β-arrestin-2-EGFP, 6 μg of sst2A, or 6 μg of sst3, and either 4 μg of GRK2 or 4 μg of empty vector. The distribution of β-arrestin was visualized sequentially in the same live cells before (0 min) and after (3, 5, 10, and 30 min) the addition of SS-14 to the culture medium. Receptor expression levels were between 1,500 and 2,000 fmol/mg membrane protein. Shown are representative images from one of three independent experiments performed in duplicate. Arrowheads, translocation of β-arrestin-2-EGFP to the plasma membrane; arrows, trafficking of β-arrestin-2-EGFP into early endosomes. Scale bar, 20 μm. C. HEK 293 cells were transfected with sst2A, sst3, or sst4 and either GRK2 or empty vector. Two days later, cells were either not exposed or exposed to 1 μM SS-14 for 20 min, and whole cell receptor phosphorylation was determined as described under “Experimental Procedures.” Upper panel, autoradiographs from representative experiments are shown. Lower panel, aliquots of the immunoprecipitates (IP) were immunoblotted with T7 antibody (IB, immunoblot) to confirm equal loading of the gels. Two additional experiments gave similar results. Note that overexpression of GRK2 strongly increased agonist-induced phosphorylation of sst2A (179 ± 25%, p < 0.05) compared with agonist-induced phosphorylation in the absence of overexpressed GRK2, whereas agonist-induced phosphorylation of sst3 (113 ± 18%) was not significantly increased in the presence of overexpressed GRK2. The position of molecular mass markers are indicated on the left (in kDa).
In the present study, we reveal previously unappreciated differences in \( \beta \)-arrestin mobilization and endosomal sorting of the five somatostatin receptor subtypes. Based on their \( \beta \)-arrestin-dependent trafficking patterns, we identify the \( sst_3 \) and \( sst_5 \) somatostatin receptors as class A receptors and the \( sst_2 \) receptor as class B receptor. On the contrary, the \( sst_3 \) receptor underwent pronounced down-regulation, whereas the \( sst_2 \) receptor linked to multiple ubiquitin monomers. In contrast, the \( sst_3 \) receptor undergoes pronounced down-regulation, which became clearly detectable after 3 h of agonist exposure (65 ± 9% of untreated control) (Fig. 7B). The degradation of \( sst_3 \) was blocked completely by the lysosomal inhibitor chloroquine (Fig. 7C). Surprisingly, the degradation of \( sst_3 \) was also blocked by the proteasomal inhibitor MG132 (Fig. 7C). Although MG132 is a highly potent inhibitor of a number of proteasome-associated proteases, this compound is not specific for proteasomes and also potently inhibits various cysteine proteases and cathepsins (25). These results suggest that \( sst_2 \) and \( sst_3 \) undergo differential endosomal sorting. Although \( sst_3 \) was sequestered into the endosomal recycling compartment, a large proportion of endocytosed \( sst_3 \) receptors was sorted into the degradative lysosomal pathway. To establish a molecular basis for the differential endosomal sorting of the \( sst_2 \) and the \( sst_3 \) receptors, we examined the agonist-dependent ubiquitination of these receptors. Cells were exposed to SS-14, receptor proteins were immunoprecipitated, and ubiquitinated receptors were detected using an anti-ubiquitin monoclonal antibody. As depicted in Fig. 8, the \( sst_3 \) receptor underwent time-dependent ubiquitination, which was apparent as a broad high molecular weight band that represents the receptor linked to multiple ubiquitin monomers. In contrast, SS-14 treatment of \( sst_3 \)-expressing cells did not result in any detectable change in \( sst_2 \) receptor ubiquitination.

**DISCUSSION**

In the present study, we reveal previously unappreciated differences in \( \beta \)-arrestin mobilization and endosomal sorting of the five somatostatin receptor subtypes. Based on their \( \beta \)-arrestin-dependent trafficking patterns, we identify the \( sst_3 \) and \( sst_5 \) somatostatin receptors as class A receptors and the \( sst_2 \) somatostatin receptor as class B receptor. On the contrary, the trafficking of the \( sst_2 \) and \( sst_3 \) somatostatin receptors did not
appear to depend on β-arrestins. We also show that sst 2A and sst 3 underwent differential endosomal sorting. In contrast to what would be expected from their β-arrestin trafficking pattern, the sst 2A receptor was rapidly recycled to the plasma membrane, whereas the sst 3 receptor was subject to ubiquitin-dependent lysosomal degradation. Using a series of truncated sst 2A receptors, we demonstrate that the molecular determinants for the formation of stable complexes between β-arrestin and sst 2A are contained within clusters of phosphate acceptor sites at the end of its cytoplasmic tail. Such clusters of phosphate acceptor sites are defined as serine/threonine residues occupying three consecutive positions or three of four positions (17). For many class B receptors, these clusters are remarkably conserved in their position within the carboxyl-terminal domain and serve as primary sites of agonist-dependent receptor phosphorylation. It should be noted that not only the sst 2A receptor but also the sst 3 and sst 4 receptors contain such clusters of phosphate acceptor sites. However, with regard to the conserved NPILY motif these clusters occupy more downstream positions in the sst 3 and sst 4 receptors than in the sst 2A receptor. We also demonstrate that the phosphorylation of the sst 2A receptor but not of the sst 3 and sst 4 receptors strongly depends on the cellular complement of GRK2.

The sst 3 receptor formed complexes with β-arrestin that dissociated at or near the plasma membrane. Neither β-arrestin mobilization nor phosphorylation of the sst 3 receptor was changed by overexpression of GRK2. Interestingly, it has previously been shown that replacement of four serine and threonine residues by alanine diminishes agonist-induced phosphorylation and internalization of the sst 3 receptor (24). Consistent with the present findings, these residues are localized upstream to the putative GRK2 phosphorylation cluster of sst 3.

In contrast to the sst 2A and sst 3 receptors, the sst 1 and sst 4 receptors failed to induce β-arrestin translocation to the plasma membrane following agonist treatment. These results imply either peculiar mechanisms of receptor desensitization or resistance to desensitization. In fact, it has previously been shown that the rat sst 4 receptor fails to internalize after agonist activation (13). Consistent with the previous findings, we show that sst 4 did not undergo any detectable phosphorylation after agonist activation despite the presence of a number of potential phosphate acceptor sites including a cluster of threo-
heterologous expression in HEK 293 cells, a large proportion of receptor protein remains in an as yet unidentified intracellular vesicular compartment (4). Thus, the fact that only a fraction of cellular sst1 receptors is targeted to the plasma membrane may explain that agonist exposure of this receptor did not promote efficient β-arrestin translocation under these conditions.

In sst2-transfected HEK 293 cells, SS-14 treatment induced β-arrestin translocation to the plasma membrane. However, this effect was only transient and less pronounced than that observed for the sst2A and sst3 receptors. Given that sst2 has a higher affinity for SS-28 than for SS-14, the limited recruitment of β-arrestin-2 could be because of limited activation of the sst2 receptor by SS-14 (26). We therefore performed β-arrestin mobilization assays using the non-peptide sst2 agonist L-817,818. The fact that very similar results were obtained under these conditions confirms that the sst2 receptor exhibits a class A receptor-like trafficking pattern.

It has been suggested recently that distinct intracellular trafficking patterns of β-arrestin determine the fate of internalized GPCRs (19, 20). Many class A receptors (e.g. μ-opioid, β2, and α1B adrenergic, endothelin A, and dopamine D1A receptors) have been shown to recycle rapidly, whereas class B receptor-like trafficking patterns are often observed for slowly recycling receptors (e.g. substance P, angiotensin AT1a, neurotransin-1, and vasopressin-2 receptors) (20). However, recent work shows that not all GPCRs fit into this classification, i.e. the N-formyl peptide receptor requires β-arrestin for its recycling but not for its internalization (27). Here, we show the sst2A receptor was rapidly recycled to the plasma membrane without any detectable loss of cellular sst2A receptors even during prolonged agonist exposure. In contrast, a large proportion of internalized sst3 receptors was ubiquitinated and sorted into the degradative lysosomal pathway, resulting in a rapid down-regulation of cellular sst3 receptors. Thus, our findings suggest that endosomal sorting of sst2A and sst3 was regulated by differential ubiquitination rather than differential β-arrestin binding of these receptors.

In conclusion, we provide evidence for differential β-arrestin-dependent trafficking and endosomal sorting of somatostatin receptor subtypes. The high density of somatostatin receptors on human neuroendocrine tumors has allowed the development of somatostatin receptor scintigraphy for tumor imaging as well as somatostatin receptor-targeted radiotherapy (9, 10). The effectiveness of these diagnostic and therapeutic manipulations strongly depends on receptor internalization and recycling. Thus, a sst2A-expressing tumor would be expected to take up radiolabeled somatostatin analogs more efficiently than tumors that express predominantly sst3 or sst1. In addition, differences have been observed in the response of neuroendocrine tumors to long-term application of stable somatostatin analogs. In patients with growth hormone-secreting pituitary adenoma, the inhibitory effects of somatostatin analogs on hormone secretion persist for many years during long-term treatment. In contrast, carcinoids are very likely to undergo desensitization within weeks to months of octreotide exposure. Although sst2A is most frequently detected, neuroendocrine tumors often express distinct patterns of somatostatin receptor subtypes (3). Thus, the differential intracellular sorting of somatostatin receptors may provide important clues about the regulation of receptor responsiveness during long-term administration of somatostatin analogs.

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