

Novel insights in somatostatin receptor physiology

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Abstract

The experimental data reviewed in the present paper deal with the molecular events underlying the agonist-dependent regulation of the distinct somatostatin receptor subtypes and may suggest important clues about the clinical use of somatostatin analogs with different pattern of receptor specificity for the *in vivo* targeting of tumoral somatostatin receptors. Somatostatin receptor subtypes are characterized by differential β -arrestin trafficking and endosomal sorting upon agonist binding due, at least in part, to the differences in their C-terminal tails. Moreover, the subcellular expression pattern of somatostatin receptor subtypes and their activity in response to agonist treatment are affected by intracellular complements, such as proteins involved in intracellular vesicle trafficking. Different somatostatin analogs may induce distinct conformations of the receptor/ligand complex, preferentially coupled to either receptor signaling or receptor endocytosis.

European Journal of Endocrinology 156 S3–S11

Somatostatin is a neuropeptide that regulates neurotransmission in the brain and hormone secretion from anterior pituitary, pancreas and endocrine cells within the gastrointestinal tract. The two physiologically active forms of somatostatin, somatostatin-14 and somatostatin-28, mediate their actions by interacting with a family of somatostatin receptors. Five genes encoding six different somatostatin receptor subtypes (sst1, sst2A and sst2B, sst3, sst4 and sst5) have been cloned (1). All somatostatin receptors belong to the superfamily of G-protein-coupled receptors (GPCRs) and have a complex overlapping pattern of expression (1, 2). Cortistatin is a neuropeptide that displays strong structural similarity to somatostatin which binds and activates somatostatin receptors (3).

The high density of somatostatin receptors on human neuroendocrine tumors has been used 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. Moreover, it has allowed the development of somatostatin receptor scintigraphy for tumor imaging as well as somatostatin receptor-targeted radiotherapy (4–7). Octreotide and lanreotide, the stable somatostatin analogs currently available for clinical use, bind only to sst2 with high affinity and to sst3 and sst5 with

moderate affinity (1). Compounds displaying high selectivity for distinct somatostatin receptor subtypes and multi-ligand somatostatin analogs, like SOM230 and BIM-23244, displaying high affinity for multiple receptor subtypes have been tested in preclinical studies (8–12).

In recent years, a number of studies have investigated the regulation of somatostatin receptor responsiveness upon agonist exposure. Due to the different levels of expression of the distinct somatostatin receptor subtypes among tumors, the comparison of their property to undergo agonist-induced desensitization and internalization and their subcellular distribution may suggest important clues about the clinical use of somatostatin analogs with different patterns of receptor specificity for the *in vivo* targeting of tumoral somatostatin receptors (4, 5, 13, 14).

The aim of the present work is to summarize experimental data dealing with the molecular events underlying the agonist-dependent regulation of somatostatin receptor subtypes sst1–3 and sst5, which play a major role in relation to the *in vivo* targeting of endocrine tumors with somatostatin analogs. In detail discussion will be focused on the following items:

- Agonist-dependent desensitization and internalization of somatostatin receptors in various cell types
- Differential trafficking and endosomal sorting of the distinct somatostatin receptors after agonist binding
- Molecular determinants within the cytoplasmic domains of somatostatin receptors and intracellular

This paper was presented at a symposium held at the Erasmus Medical Center, Rotterdam, The Netherlands, 2005. The symposium was jointly organized by LJ Hofland, Erasmus Medical Center, and A Colao, Federico II University of Naples, Italy. Ipsen partially supported the publication of these proceedings.

complements involved in the regulation of somatostatin receptor responsiveness

- Differential effects of distinct somatostatin analogs on receptor signaling and endocytosis.

A paradigm about agonist-dependent regulation of GPCRs

A common property of most GPCRs is that ligand treatment decreases receptor responsiveness through a mechanism called desensitization (Fig. 1). After agonist binding and activation of GPCRs, the signaling is turned off by phosphorylation of intracellular receptor domains by G-protein-coupled receptor-kinases (GRKs) and subsequent recruitment of cytoplasmic proteins termed arrestins that interrupt coupling between the receptor and its cognate heterotrimeric G-protein (15–17). This mechanism is usually followed by dynamin-dependent endocytosis of receptor/ligand complexes (internalization), which was demonstrated to play a key role in receptor down-regulation and resensitization (18–20). β -arrestins also function as docking proteins that link the receptor to the component of the endocytic machinery such as AP-2 and clathrin (21). However, arrestins do not mediate endocytosis of all GPCRs (22). Within the endosomal compartment, β -arrestins regulate the rate at which internalized receptors are dephosphorylated and recycled to the plasma membrane (23, 24). Based on their binding properties to different isoforms of arrestins, Oakley and coworkers categorized GPCRs into two classes. Class A receptors (e.g. μ -opioid, β_2 and α_{1B} adrenergic, endothelin and dopamine D1A receptors) do not bind visual arrestin and have a higher affinity for

non-visual β -arrestin-2 than β -arrestin-1. Class B receptors (e.g. substance P, angiotensin AT_{1b}, 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 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 it does not internalize with them. For class B receptors, β -arrestin forms stable complexes with the receptors such that the receptor– β -arrestin complex is internalized as a unit into early endosomes (17).

The C-terminal tail of GPCRs plays an important role in the early molecular events that lead to receptor desensitization and sequestration. Formation of stable complexes between β -arrestin and GPCRs strongly depends on the presence of clusters of phosphate acceptor sites (defined as serine or threonine residues occupying three consecutive positions or three of four positions) within the carboxyl-terminal tail of the receptor (16, 17). These clusters serve as primary sites of agonist-dependent receptor phosphorylation and are remarkably conserved in their position downstream of the highly conserved NPILY motif, which marks the end of the seventh transmembrane domain and the beginning of the cytoplasmic tail of GPCRs.

Agonist-dependent regulation of somatostatin receptors

The distinct receptor subtypes differ at their C-terminal domains for the presence of clusters of putative phosphate acceptor sites and for the position of these clusters with regard to the NPILY motif and the end of

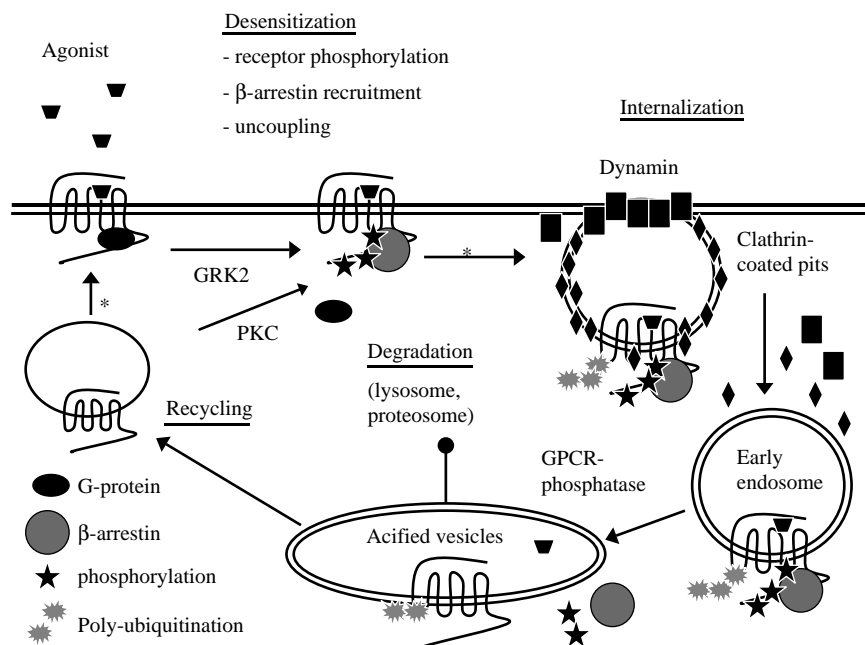


Figure 1 Intracellular complements involved in GPCR desensitization, internalization and intracellular trafficking, following agonist binding. Adapter and scaffolding proteins (i.e. amphiphysins and PDZ-domain containing proteins) may be involved in the regulation of both receptor endocytosis (*) and receptor targeting to the plasma membrane (*) and ultimately influence the subcellular receptor localization. β -arrestin dissociation from the receptor is believed to influence the rate at which internalized receptors are dephosphorylated and recycled to the plasma membrane. Receptor-ubiquitination is a covalent modification that may direct the receptor molecule to proteosomal or lysosomal degradation.

the chain (Fig. 1). Based on the previously mentioned Oakley's studies (16, 17), these differences may dictate differential trafficking and endosomal sorting of somatostatin receptors after agonist binding.

Somatostatin receptor subtype-1 (*sst1*)

Species-related differences in agonist-mediated endocytosis of *sst1* have been observed. Agonist exposure induced the desensitization of adenylyl cyclase (AC) inhibition in transfected host cells expressing human *sst1* (h-*sst1*) or rat *sst1* (r-*sst1*) (25–28). However, h-*sst1* but not r-*sst1* failed to internalize in an agonist-dependent manner (26, 29, 30). In agreement with this observation, a low-efficiency sequestration of fluorescent somatostatin was observed in COS cells expressing h-*sst1* (31). Rat *sst1* was demonstrated to conspicuously internalize in transfected human embryonic kidney (HEK293) cells (30). In Chinese hamster ovary (CHO) cells, its internalization was reported to be moderate and slow implying that the internalization is also dependent upon the cellular environment. Incubation of cells with somatostatin also caused a rapid increase in r-*sst1* phosphorylation (28). Recently, we employed functional β -arrestin-1 and β -arrestin-2 conjugated to enhanced green fluorescent protein (eGFP) to visualize the translocation of β -arrestins to the plasma membrane in live HEK293 cells transfected with distinct rat somatostatin receptor subtypes using confocal imaging approaches (32). In cells expressing r-*sst1*, the addition of saturating concentrations of somatostatin to the medium did not induce the translocation of β -arrestin-1 or β -arrestin-2 to the plasma membrane, suggesting that r-*sst1* desensitization and internalization do not depend on β -arrestin recruitment. Alternatively, it should be noted that upon overexpression of h-*sst1* in transfected HEK293 cells, a large proportion of receptor proteins remains in an as yet unidentified intracellular vesicular compartment (33). The fact that only a fraction of cellular *sst1* is targeted to the plasma membrane may explain that agonist exposure of this

receptor did not promote efficient β -arrestin translocation under these conditions.

Kumar *et al.* reported that h-*sst1* is up-regulated in stably transfected CHO-K1 cells upon somatostatin exposure (26). The same research group showed that h-*sst1* up-regulation after long-term agonist treatment is critically dependent on a specific Lys³⁵⁹–Ser³⁶⁰–Arg³⁶¹ sequence motif within the receptor's C-tail and requires interactions with β -arrestin-1 and calcium-calmodulin protein kinases. It seems to be the result of re-targeting of a pre-existing pool of receptors from the Golgi apparatus to the plasma membrane. Interestingly, a synthetic non-peptidic subtype-selective analog for *sst1* was unable to trigger h-*sst1* up-regulation (29).

Previously cited data suggest species-related differences in β -arrestin trafficking of *sst1*, in that r-*sst1* does not recruit β -arrestin-1 upon agonist exposure (32), whereas β -arrestin-1 overexpression is able to influence agonist-induced internalization and up-regulation of h-*sst1* (29). Although β -arrestin binding to h-*sst1* needs to be elucidated further and the use of two different cell models (HEK293 vs CHO-K1 cells) makes it difficult to compare the results between the two studies, it is worth noticing that h-*sst1* and r-*sst1* differ at their C-terminal tail for the presence of a cluster of putative phosphate acceptor sites (Thr³⁸³–Cys³⁸⁴–Thr³⁸⁵–Ser³⁸⁶) in h-*sst1*. In r-*sst1*, Ser³⁸⁶ residue is replaced by alanine (Fig. 2) and this amino acid substitution might account for a lower affinity for β -arrestins of r-*sst1* vs h-*sst1*.

Somatostatin receptor subtype-2 (*sst2A* and *sst2B*)

There is ample evidence for agonist-induced desensitization and/or internalization of the *sst2* subtype as assessed in several cell lines and demonstrated in the nervous tissue (2, 34–37). Both *sst2* splicing isoforms were reported to desensitize following agonist exposure (38).

sst1 (CAA44193 and NP_001040)

r:³¹⁹NPILYGFLSDNFKR\$FQRILCL\$WMDNAAEPPVDYYATALKSRAY\$VEDFQPENLE\$GGVFRNG
TCASRISTL
h:³¹⁹NPILYGFLSDNFKR\$FQRILCL\$WMDNAAEPPVDYYATALKSRAY\$VEDFQPENLE\$GGVFRNG
TCTSRITTL

sst2A (NP_062221 and NP_001041)

r/h:³⁰⁸NPILYAFLSDNFKK\$FQNVLCVVKV\$GAEDGERS\$D\$KQDK\$SRLNETTTETQRTLLNGDLQTSI
sst2B (Schindler M *et al.* Br. J. Pharm. 1998, 125 pp 209–217)
r:³⁰⁸NPILYAFLSDNFKK\$FQNVLCVVKADNSKTGEEDTMAWV

sst3 (NP_598206 and AAP32288)

r:³¹⁸NPILYGFLSYRFKQGFRRILLRPSRRVR\$QEPG\$GPPPEKTEEEEDDEEEERREEEERRMRQGEQEMNGR
L\$QIAQPGP\$GQQQRPC\$TGTAKEQQLLPQEA\$TAGDKASTL\$SHL
h:³⁰⁹NPILYGFLSYRFKQGFRRVLLRPSRRVR\$QEP\$TVGPPPEKTEEEEDDEEEDGEE\$REGGK\$KEMNGRVS
QITQPGT\$GQERPP\$RVA\$KEQQLLPQEA\$TGEK\$STMRISYL

sst5 (NP_037014 and NP_001044)

r:³⁰⁰NPILYGFLSDNFRQ\$FRKVLCLRRGYGMEADAI\$EPRDPK\$GRPQATLPT\$RSC\$EANGLMQTSRI
h:³⁰⁰NPVLYGFLSDNFRQ\$FQKVLCLRKG\$GAKDADATEPRPDRIRQQEATPPA\$HRAAANGLMQTSKLL

Figure 2 Carboxyl-terminal amino acid sequences of cloned rat and human somatostatin receptor subtypes are depicted beginning with the conserved NPILY motif, which marks the end of the seventh transmembrane domain and the beginning of the cytoplasmic tail. PubMed accession numbers for the full sequences are reported within brackets. Putative palmitoylation sites (Cys residues) and potential phosphate acceptor sites are underlined; putative clusters of phosphate acceptor sites are underlined twice.

Saturating concentrations of somatostatin concomitantly induced sst2A desensitization and phosphorylation in transfected GH4C1 cells overexpressing the receptor (39). Agonist-induced phosphorylation of sst2A does not appear to be dependent on protein kinase C (PKC) activation. Indeed, incubation of cells with phorbol 12-myristate 13-acetate was shown to stimulate receptor phosphorylation and to potentiate receptor internalization (40). In HEK293 cells, a T7-tagged r-sst2A construct lacking 20 amino acids of the C-terminal tail was shown to undergo agonist-independent constitutive internalization without being phosphorylated. Moreover, a truncated form of the receptor lacking the last 44 amino acids from the C-terminus was efficiently internalized after agonist activation without being phosphorylated (41). Seemingly, phosphorylation of the C-terminal domain of sst2A is not required for the receptor internalization in all cellular environments. Elberg *et al.* (42) investigated the homologous and heterologous regulation of endogenous sst2-phosphorylation and -internalization in AR42J rat pancreatic acinar cells upon stimulation by somatostatin or by cholecystokinin and bombesin. Both somatostatin and heterologous hormones stimulated sst2 phosphorylation by PKC-independent and -dependent mechanisms respectively. Phosphorylation sites were in the third intracellular loop and in the C-terminal tail of the receptor. Bombesin and cholecystokinin also induced receptor internalization by a mechanism that was independent of the receptor phosphorylation. In fact, their effect on sst2 internalization was not blocked by a PKC inhibitor. Finally, in contrast to the extensive desensitization of sst2 following the exposure to somatostatin, heterologous desensitization was modest although significant.

Little is known about GPCR phosphorylation *in vivo*. Liu *et al.* (43) investigated the phosphorylation state of h-sst2A in human neuroendocrine tumors using a newly developed gel-shift assay. The receptor from a somatostatinoma was completely phosphorylated. In contrast, only unphosphorylated sst2A was present in tumors that did not express somatostatin mRNA and so were not exposed to autocrine stimulation. Immunocytochemical localization showed that phosphorylated receptor was mostly intracellular, whereas unphosphorylated receptor was localized at the cell surface.

In both HEK293 and CHO-K1 cells co-transfected with β -arrestin-eGFP and r-sst2A, the type of interaction between β -arrestins and r-sst2A resembled that of a class B GPCR in that, upon receptor activation, β -arrestin and the receptor formed stable complexes and internalized together into the same endocytotic vesicles (32, 37). Using a series of truncated mutants of r-sst2A lacking either 10, 15, or 20 amino acids from the C-terminus and a mutant in which Thr^{353,355,356} (Fig. 2) were replaced by alanine residues, we demonstrated that many, if not all, of the potential acceptor sites contained within the 20 C-terminal

amino acids of r-sst2A are involved in GRK-2-mediated phosphorylation of the receptor, and that phosphorylation of these sites is required for formation of stable β -arrestin-sst2A complexes. The cluster of Thr^{353,355,356} is crucial for β -arrestin binding and may represent the primary site for GRK-2-mediated phosphorylation (32). Although β -arrestin is endocytosed with the receptor after somatostatin stimulation, 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 (44).

The two splice isoforms of sst2, namely sst2A and sst2B, differ at their C-terminus (Fig. 2). The sst2B may resemble a truncated mutant of sst2A lacking the cluster of threonine residues in positions 353–356. Interestingly, in CHO-K1-transfected cells, mouse sst2B mediated a more efficient inhibition of AC and was revealed to be more resistant to agonist-induced reduction of binding than the longer isoform (38).

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. Recently, we examined the redistribution of r-sst2A in HEK293 cells after agonist-induced internalization, using a confocal imaging approach (32). Nearly the entire pool of internalized r-sst2A recycled to the plasma membrane during 40 min incubation in the absence of somatostatin. We also observed a high degree of colocalization of sst2A with transferrin, a well-established marker of early and recycling endosomes (45). Studies of subcellular localization of sst2A in the dendrites of cortical neurons of octreotide-injected rats revealed rapid and massive endocytosis of the receptor through a clathrin-dependent pathway and colocalization of sst2A immunoreactive endosomes with transferrin receptor (35). Experiments performed in Neuro2A neuroblastoma cells and in CHO cells expressing h-sst2A suggested that the stable somatostatin analog ¹²⁵I-BIM-23027 can cycle continuously between the cell surface and the intracellular compartment (46, 47). However, in CHO cells expressing m-sst2A, a low-efficient recycling of the receptor after agonist-induced internalization has been shown (44). We assessed the downregulation of r-sst2A after prolonged somatostatin exposure in HEK293 cells and did not observe any detectable change in the level of cellular sst2A during the 16 hour treatment period. In summary, different works suggest that sst2A, unlike the class B receptor, is efficiently recycled to the plasma membrane and does not enter any degradative pathway.

In both transfected and neuroendocrine cells expressing the receptor endogenously, sst2A is almost exclusively confined to the cell surface in the absence of the agonist (32, 37, 48). The trans-Golgi network (TGN) is a dynamic organelle through which nascent secretory and transmembrane proteins are sorted and packaged into carrier vesicles for transport to either the

plasma membrane or the endosomal compartments. Seemingly, newly synthesized sst2A is rapidly and completely targeted to the plasma membrane. Recently, Sarret *et al.* investigated the role of amphiphysin IIb in cellular trafficking of sst2A receptor in mouse pituitary corticotrope cells (AtT-20). Amphiphysin II belongs to a family of SH3 domain-containing proteins which are thought to function as adapter proteins that cooperate with the recruitment of components of the endocytotic machinery. Seven different splice isoforms of amphiphysin II have been isolated. In particular, amphiphysin IIb, which is lacking an important determinant for plasma membrane targeting and does not bind clathrin, may have functions unrelated to endocytosis. In transfected AtT-20 cells overexpressing amphiphysin IIb, the endogenous sst2A was sequestered intracellularly within the TGN even in the absence of agonist stimulation, as documented by fluorescent immunolabeling of the receptor, and the inhibitory effect of somatostatin on corticotropin-releasing-factor induced adrenocorticotrophic hormone (ACTH) secretion was significantly reduced when compared with wild type cells, in keeping with the decrease in the density of cell surface sst2A (48). The previous results suggest that amphiphysin IIb is involved in receptor targeting from the TGN to the plasma membrane and its level of expression may affect the cellular responsiveness to pharmacological treatments.

Biochemical, biophysical and functional studies have shown that GPCRs can assemble on the plasma membrane as homo- and heterodimers. In stably transfected cells, ligand activation of h-sst2 resulted in the dissociation of self-associated h-sst2 homodimers to monomers. Remarkably, inhibiting receptor dissociation by a cell-impermeable cross-linking agent prevented agonist-induced receptor internalization (49). As far as rat sst2A is concerned, self-associated receptor homodimers were shown by western blot in transfected HEK293 cells, but agonist treatment did not have a considerable effect on dimer dissociation (50). Conversely, in HEK293 cells co-expressing r-sst2A and r-sst3, agonist treatment was able to induce the dissociation of r-sst2A/r-sst3 heterodimers and the selective internalization of sst2A (50). In transfected HEK293 cells, r-sst2A also forms heterodimers with μ -opioid receptor, a member of a GPCR family closely related to somatostatin receptors. Exposure of the cells to a sst2A selective ligand induced phosphorylation, internalization and desensitization of sst2A as well as μ -opioid receptor. Similarly, the activation of μ -opioid receptor by a selective agonist promoted phosphorylation and desensitization of both μ -opioid and sst2A receptor. Actually, cross-phosphorylation of sst2A leads to the separation of the dimer at the plasma membrane and sst2A was not internalized (51).

The sst2 is the most widely distributed somatostatin receptor in normal tissues and tumors. Receptor-mediated ligand internalization has been used to

catalyze the accumulation of stable, radiolabeled somatostatin analogs in tumor cells that express the sst2 receptor (6, 13). Such radioligand accumulation allows tumor imaging by gamma camera scintigraphy (52) and tumor treatment by peptide receptor-targeted radiotherapy (6). Recent studies in transfected host cells expressing sst2 receptor documented that different somatostatin analogs may differentially regulate sst2 receptor signaling and endocytosis (5, 37, 53). Upon ligand binding, sst2 internalization was triggered by receptor activation. Somatostatin antagonists were unable to induce receptor internalization and effectively blocked the agonist-induced internalization (37, 53). Actually, somatostatin agonists may differ in their relative potencies for stimulation of the sst2 receptor signaling and endocytosis. For example, [Tyr³]-octreotide was more potent than somatostatin-14 at inhibiting cAMP production, whereas the two peptides showed similar potencies at inducing receptor internalization. The non-peptide ligand L-779,976 binds to sst2 with the same affinity as somatostatin-14, but it was 10 times more potent than somatostatin-14 in inducing cAMP inhibition and only twice as potent in stimulating receptor endocytosis. Moreover, at maximal doses, L-779,976 was unable to produce more than 70% of the somatostatin-induced receptor internalization. In contrast to somatostatin, L-779,976 induced a transient translocation of β -arrestin-2-GFP to the plasma membrane of sst2-expressing CHO cells. The β -arrestin-receptor complex dissociated before receptor internalization, suggesting a lower affinity of β -arrestin for the receptor conformation induced by L-779,976 as compared with the somatostatin-induced conformation (37). All these data provide evidence for distinct agonist-induced sst2 receptor conformations preferentially coupled to a different functional pathway.

The immunocytochemical internalization assay using transfected HEK293 and CHO-K1 cells expressing sst2 has been recently applied to compare the relative potencies *in vitro* of a number of chelated somatostatin analogs and iodinated, sugar-containing octreotide analogs, for use in nuclear medicine. All the analogs tested were able to efficiently induce sst2 internalization with different relative potencies (5).

Somatostatin receptor subtype-3 (sst3)

In transfected HEK293 and rat insulinoma (RIN) cells expressing r-sst3, somatostatin preincubation reduced agonist-mediated inhibition of forskolin-stimulated cAMP levels (27, 54). Both human and rat sst3 were found to internalize efficiently after agonist stimulation. As for the sst2A subtype, internalization of r-sst3 was mediated through a clathrin-dependent pathway since it was prevented by hyperosmolar sucrose and phenylarsine oxide (25, 27, 30, 54); in transfected RIN cells, clathrin colocalized with r-sst3 during receptor internalization (55). The r-sst3 is phosphorylated in

response to the agonist treatment and its C-terminal tail contains several putative phosphate acceptor sites. Four hydroxyl amino acid residues in the C-terminus (Ser³⁴¹, Ser³⁴⁶, Ser³⁵¹ and Thr³⁵⁷) have been identified as primary sites of agonist-dependent receptor phosphorylation. These four residues are also required for the agonist-induced receptor desensitization and internalization (54). In HEK293-transfected cells, sst3 phosphorylation was largely independent of the presence of the overexpressed GRK-2 (32).

In HEK293-transfected live cells, the pattern of interaction between β -arrestin-eGFP and r-sst3 resembled that of a class A GPCR. The addition of somatostatin induced a rapid redistribution of β -arrestin-1 as well as β -arrestin-2 from the cytoplasm to the plasma membrane. Actually, fluorescence outlining the shape of the cells was less marked in cells cotransfected with β -arrestin-1 when compared with cells cotransfected with β -arrestin-2, suggesting that r-sst3 has a higher affinity for β -arrestin-2 than β -arrestin-1. After prolonged agonist exposure, β -arrestins redistributed uniformly to the cytoplasm suggesting that the β -arrestin/r-sst3 complex dissociated before receptor internalization. In the absence of the agonist, r-sst3 was almost exclusively confined to the plasma membrane. After 30 min of somatostatin exposure, the receptor was extensively sequestered into intracellular vesicles. In contrast to that observed for r-sst2A, after agonist withdrawal only part of the internalized sst3 was redistributed to the plasma membrane, whereas a large proportion of sst3 was sequestered into intracellular clusters of relatively large vesicles. Studies of colocalization of internalized receptors with transferrin evidenced that both sst2A and sst3 were initially internalized through the same endocytic pathway and colocalized with transferrin, a marker of early and recycling endosomes. The sst3 was then sorted, at least in part, to a population of vesicles that did not contain transferrin and were probably distinct from those constituting the recycling pathway. Moreover, r-sst3 underwent pronounced down-regulation, which became apparent after 3 h of agonist exposure. The degradation of sst3 was blocked completely by the lysosomal inhibitor chloroquine and the proteasomal inhibitor MG132. The differential endosomal sorting of r-sst2A and r-sst3 was mediated by differential ubiquitination of the two receptor subtypes. After agonist exposure, r-sst3 was immunoprecipitated as a broad high molecular band that represented the receptor linked to multiple ubiquitin monomers, whereas no detectable change in sst2A receptor ubiquitination was observed (32). In rat neuroendocrine insulinoma cells co-expressing β -arrestin-1-eGFP and r-sst3, a stable interaction between r-sst3 and β -arrestin-1 was observed. Stimulation with somatostatin induced trafficking of β -arrestin-1-eGFP and r-sst3 to colocalize into the same endocytotic vesicles. Co-expression of a dominant

negative mutant of β -arrestin with the receptor blocked the internalization of sst3 (55). Seemingly, the type of interaction between sst3 and β -arrestins is also dependent upon the cellular environment.

Somatostatin receptor subtype 5 (sst5)

The sst5 is the only somatostatin receptor subtype with a greater affinity for somatostatin-28 than somatostatin-14.

In CHO-transfected cells, h-sst5 functionally desensitizes in response to agonists (56, 57). The cysteine residue which occupies position 320, a putative palmitoylation site, appeared to be crucial for uncoupling from AC and internalization of the activated-receptor/ligand complex (56).

In transfected HEK293 cells expressing r-sst5, we observed that somatostatin-14 treatment induced translocation of fluorescent β -arrestin-2 but not β -arrestin-1 to the plasma membrane. However, even for β -arrestin-2, the effect was only transient and less pronounced than that observed in cells expressing r-sst2A or r-sst3. The limited recruitment of β -arrestins could be related to limited activation of sst5 by somatostatin-14. We therefore performed β -arrestin mobilization assays by the non-peptide sst5 agonist L-817, 818. The fact that very similar results were obtained under these conditions confirmed that sst5 exhibits a class A receptor-like trafficking pattern (32). The lack of clusters of phosphate acceptor sites within the C-terminal domain of r-sst5 is consistent with the low stability of the interaction between the receptor and β -arrestins.

Both r-sst5 and h-sst5 efficiently internalize upon stimulation of somatostatin-28, whereas somatostatin-14 did not reduce the number of receptor molecules on the cell surface of HEK293 and RIN cells expressing sst5 (5, 27, 30). Recently, Cescato and coworkers showed that, unlike the native ligand somatostatin-28, several high affinity synthetic sst5 agonists such as L-817,818, BIM-23244, KE108 and chelated somatostatin analogs were unable to elicit sst5 internalization in transfected HEK293 cells (5). These data provide evidence for differential effects of sst5 agonists on receptor signaling and endocytosis and suggest a lower efficacy of sst5 receptors as compared with sst2 receptors in mediating the uptake of radiolabeled somatostatin analogs by tumor cells though the internalization of the receptor–ligand complex (see sst2 paragraph). However, in COS cells expressing r-sst5, confocal microscopy analysis revealed that a fluorescent somatostatin analog was efficiently internalized and clustered towards the center of the cell, whereas the receptor immunoreactivity remained associated with the plasma membrane. The availability of cell surface receptors during the course of exposure to a somatostatin analog was attributed to efficient receptor recycling and to mobilization of receptors from intracellular stores to the cell surface (58).

The existence of an intracellular pool of sst5 receptors has also been documented by immunocytochemical analysis of the distributional pattern of endogenous sst5 in mouse AtT-20 cells in the absence of agonist. Immunoreactive sst5 receptors were observed both at the cell surface and in a prominent cytoplasmic pool, whereas sst2 immunoreactivity was almost completely confined to the plasma membrane under the same conditions. In amphiphysin IIb-transfected AtT-20 cells, sst5 immunoreactivity, as well as sst2 immunoreactivity (see sst2 paragraph for details), was selectively concentrated in a juxtannuclear compartment (48).

Recently, by yeast two-hybrid screening, Wente *et al.* have identified two proteins that are able to interact with the distal C-terminus of mammalian sst5 and to regulate its intracellular sorting (59). PIST and PDZK1 are PDZ (PSD-95/discs large/zonula occludens)-domain containing proteins. PDZ domain proteins frequently act as scaffolding proteins because of their multiple protein interaction motifs and can target a receptor to specific subcellular domains. Remarkably, all mammalian somatostatin receptor subtypes contain a potential C-terminal PDZ ligand motif (59). PIST is Golgi-associated and retains sst5 in the Golgi apparatus when co-expressed with the receptor in HEK293 cells; PDZK1 associates with sst5 at the plasma membrane. In mouse pituitary AtT-20 cells, PIST colocalized with endogenous sst5 in the Golgi apparatus. A truncated sst5 mutant lacking 12 amino acids from the C-terminus was localized to the plasma membrane when it was expressed in AtT-20 cells. These data suggest that the C-terminal PDZ ligand motif plays a pivotal role in the intracellular localization of the endogenous sst5 receptor in AtT-20 cells. Moreover, the lack of PDZ ligand motif did not interfere with agonist-dependent receptor internalization, but it inhibited receptor recycling (59).

Concluding remarks

The experimental data reviewed in the present paper suggest the following considerations:

- Somatostatin receptor subtypes are characterized by differential β -arrestin trafficking and endosomal sorting upon agonist binding due, at least in part, to the differences in their C-terminal tails
- Intracellular complements, such as proteins involved in intracellular vesicle trafficking and second messenger kinases may affect the subcellular expression pattern of somatostatin receptor subtypes and their activity in response to agonist treatment
- Different somatostatin analogs may induce distinct conformations of the receptor/ligand complex preferentially coupled to either receptor signaling or receptor endocytosis.

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Received 31 August 2006

Accepted 6 December 2006