Somatostatin receptor 5 (sst_5)-regulated trafficking of somatostatin receptor 2A (sst_{2A}) in epithelial and pituitary cell lines

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Abstract

The purpose of the present study was to characterize the mechanisms by which cells that express both sst_{2A} and sst_5 receptors remain responsive to SRIF analogs under continuous stimulation. Hypothesizing that the expression of sst_5 affects the regulation of sst_{2A}, we compared the agonist-induced internalization and intracellular trafficking of sst_{2A} receptors in cells expressing sst_{2A} alone and together with sst_5. In cells stably expressing sst_{2A}, stimulation with a non-selective SRIF agonist ([D-Trp^8]-SRIF-14) and a sst_2-selective agonist (L-779,976) induced sst_{2A} internalization and decreased responsiveness to further agonist stimulation. By contrast, in cells co-expressing sst_{2A} and sst_5, while stimulation with [D-Trp^8]-SRIF-14 similarly induced sst_{2A} internalization and loss of cellular responsiveness, L-779,976 stimulation resulted in a maintenance of surface receptor density, and consequently, continued responsiveness to [D-Trp^8]-SRIF-14. Taken together, our results suggest that sst_5 exerts an influence on the regulation of sst_{2A}, such that cells expressing these receptor subtypes are able to remain responsive to SRIF agonists under continuous exposure.

Résumé

Les cellules qui expriment sst_{2A} et sst_5 peuvent répondre à une stimulation prolongée aux analogues SRIF. Nous voulions donc caractérisé les mécanismes impliqués dans le maintien de cette réponse. Avec l'hypothèse que l'expression du récepteur sst_5 affecte la régulation de sst_{2A}, nous avons comparé l'internalisation et le trafic de sst_{2A} dans des cellules exprimant sst_{2A} ou sst_{2A} et sst_5. La stimulation des cellules exprimant sst_{2A} avec un agoniste non-sélectif (SRIF-14) ou sst_2-sélectif (L-779,976) induit l'internalisation de sst_{2A} ainsi qu'une diminution de la réponse à une stimulation subséquente. Dans les cellules qui co-expriment sst_{2A} et sst_5, la réponse au SRIF-14 demeure similaire mais la stimulation au L779,976 entraîne le maintien des récepteurs à la surface et la conservation de la capacité à répondre à une stimulation ultérieure. Ces résultats suggèrent que sst_5 influence la régulation de sst_{2A} et que les cellules qui les co-expriment peuvent répondre à l'exposition continue aux agonistes SRIF.

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List of Abbreviations

AC: adenylate cyclase

ACTH: adrenocorticotropic hormone

cAMP: cyclic 3', 5'-adenosine monophosphate

CNS: central nervous system

CRF: corticotropin releasing factor

GDP: guanosine diphosphate

GH: growth hormone

GHRH: growth hormone releasing hormone

GPCR: G protein-coupled receptor

GRK: G protein-coupled receptor kinase

GTP: guanosine triphosphate

GTPase: guanosine triphosphatase

IGF: insulin-like growth factor

mRNA: messenger ribonucleic acid

PAO: phenylarsine oxide

PKA: protein kinase A

PKC: protein kinase C

PRL: prolactin

RNA: ribonucleic acid

SRIF: somatotropin-release inhibiting factor

Sst: somatostatin receptor subtype

TGN: trans-Golgi network

TM: transmembrane

TSH: thyroid stimulating hormone

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Introduction

1. Historical Review

1.1 The Discovery of Somatostatin

It was long hypothesized that a hypothalamic hypophysiotropic releasing factor is involved in the regulation of growth hormone (GH) secretion (Barnett, 2003). Krulich and colleagues were the first to provide evidence to this effect in 1968. The purification of extracts from about 500,000 sheep hypothalamic fragments led in 1972 to the identification of somatotropin-release inhibiting factor-14 (SRIF-14 or somatostatin-14), a tetradecapeptide with a potent inhibitory effect on GH release from the pituitary gland (Brazeau *et al.*, 1973). This discovery was somewhat unexpected since a growth hormone releasing factor was the hypothesized substance. Soon thereafter, the inhibitory effect of SRIF-14 on GH release was confirmed in healthy humans (Siler *et al.*, 1973), and the peptide was localized in the hypothalamus and pituitary gland (Desy and Pelletier, 1977).

Following its initial characterization as a modulator of GH release, the various inhibitory effects of somatostatin on peripheral organs began to surface. Evidence from studies on baboons revealed SRIF-14's ability to promote hypoglycemia by inhibiting the secretion of glucagon and insulin from the pancreas (Koerker *et al.*, 1974). Subsequently, somatostatin's peripheral functions in humans were demonstrated,

including its inhibitory effects on pancreatic alpha and beta cell function (Leblanc *et al.*, 1975); its ability to suppress elevated GH, prolactin (PRL), insulin and glucose levels in patients suffering from acromegaly (Yen *et al.*, 1974), and it's ability to regulate thyroid stimulating hormone (TSH) release (Siler *et al.*, 1974). Somatostatin's influence on the gastrointestinal system was first demonstrated in cats when Gomez-Pan and colleagues (1975) showed that the neuropeptide inhibited gastric acid and pepsin secretion. However, it was not until a few years later that the N-terminally extended form of the peptide, SRIF-28, was isolated and established as the active isoform in the digestive system (Pradayrol *et al.* 1980).

Over the last 25 years, knowledge about the multifarious functions of somatostatin has evolved (Guillemin, 2005). This includes the peptide's now well established immunomodulatory activity. For example, at sites of chronic inflammation, somatostatin inhibits production of interferon-γ, tumour necrosis factor-α (TNF-α), corticotropin releasing factor (CRF) and Substance P (review in Krantic *et al.*, 2004). The anti-proliferative actions of somatostatin have made it a therapeutic candidate for the treatment of tumours (Guillemin, 2005), and somatostatin has been shown to be involved in the pathophysiological development of Alzheimer's disease, Huntington's chorea, Parkinson's disease, epilepsy and diabetes mellitus (Epelbaum *et al.*, 1994). In addition, it has been implicated as a neuromodulator that affects a variety of processes mediated by the central

nervous system (CNS) including motor coordination, sleep, nociception and attention (Patel, 1999).

1.2 The Pharmacological Characterization of Somatostatin Receptors

Somatostatin receptors were initially characterized in the rat pituitary cell line GH_4C_1 (Schonbrunn and Tashjian, 1978). Early pharmacological studies revealed the heterogeneous nature of SRIF receptors (Mandarino, 1981; Srikant and Patel, 1981a, 1981b, 1981c). Binding sites for radiolabeled SRIF-14 and SRIF-28 were localized in the central nervous system, anterior pituitary gland, gastrointestinal system and pancreas (Patel, 1999). Furthermore, pharmacological analysis using the octapeptide SRIF analog SMS 201-995 (Octreotide®) and the hexapeptide analog MK-678 suggested the expression of multiple SRIF-14 binding sites. Thus, the two initial subgroups of SRIF receptors were distinguished by their pharmacological properties: while SRIF-1 receptors bound to Octreotide® and MK678 with high affinity, SRIF-2 receptors did not.

Direct evidence for individual somatostatin receptor subtypes did not exist until the genes encoding subtypes sst_1 and sst_2 were identified by molecular cloning from human islet cells (Yamada *et al.*, 1992). Over the next several years, the cloning of sst_3, sst_4 and sst_5 originating from human, rat and mouse tissues followed (reviewed in Møller *et al.*, 2003). In total, five distinct SRIF receptor genes, each originating on a different

chromosome and encoding proteins of 356-391 amino acids in length, were identified in various tissues.

2. Cellular and Molecular Biology of the Somatostatin Receptors

2.1 Gene Transcription and Synthesis

Somatostatin receptors are members of the largest superfamily of integral membrane proteins, the G protein-coupled receptors (GPCRs), which are comprised of seven α-helical transmembrane-spanning segments, an extracellular N-terminus and a cytosolic C-terminus (Patel, 1999; Ferguson, 2001). The sst_2 receptor gene is the only one containing an intron at the 3' end of the coding sequence, which is cleaved to give rise to two splice variants: sst_{2A} (the long form) and sst_{2B} (a C-terminally truncated form) (Vanetti *et al.*, 1992). Of the two variants, sst_{2A} is thought to be the principal physiologically active form (Barnett, 2003).

Among mammalian species, SRIF receptors maintain a large degree of structural homology. For example, there is a 93-96% amino acid sequence homology between human and rodent sst_{2A} and an 82-83% homology for sst_5 (Patel, 1999). The sequence identity between somatostatin receptor subtypes ranges from 39-57%, but each receptor contains a conserved *signature* sequence in the seventh transmembrane (TM) domain, YANSCANPI/VLY. The GPCR family showing the highest sequence similarity with the somatostatin receptors is the opioid receptor family. For example, the mouse delta opioid receptor exhibits 37% sequence identity with sst_1 (Evans *et al.*, 1992), and both the delta and

kappa subtypes were originally cloned in the search to identify new somatostatin receptor subtypes (Tannenaum and Epelbam, 1999).

Following synthesis in the rough endoplasmic reticulum, SRIF receptors may undergo several possible post-translational modifications as they travel through the Golgi complex. With the exception of sst_3, the C-termini of all somatostatin receptor subtypes are anchored to the plasma membrane by palmitoylation on a cysteine residue, thus forming a fourth cytoplasmic loop (Tannenaum and Epelbam, 1999). On the N-terminal segment of each receptor subtype there are one to four sites for glycosylation (Patel, 1999). Following post-translational modification, SRIF receptors leave the Golgi apparatus via vesicles, which transport them toward the cell periphery and insert them upon fusion with the plasma membrane (Barnett, 2003). Furthermore, the C-terminal segment and the second and third intracellular loops of somatostatin receptors contain several possible motifs that may be phosphorylated.

2.2 Ligand Binding

2.2.1 Ligand Binding Domain

Biochemical analysis of SRIF receptor amino acid sequences revealed the structural basis of early pharmacological observations. All somatostatin receptors contain a ligand binding domain or "pocket" made up by TM domains III-VII (Patel, 1999). According to the model developed from Octreotide® binding to human sst_2 receptors, Asn^{276} and Phe^{294} in

TM domains V and VII, respectively, (which are not present in sst_1) are involved in hydrophobic interactions with Phe^7, Trp^8, Lys^9 and Thr^{10} on the peptide (Kaupmann *et al.*, 1995). Asp^{137} in TM domain III anchors the ligand in the binding pocket through an electrostatic interaction with Lys^9. Using site-directed mutagenesis, it was demonstrated that the substitution of Gln^{291} and Ser^{305} in sst_1 receptors for Asn^{276} and Phe^{294} corresponded to a 1000-fold increase in binding affinity for Octreotide®. These experiments provided biochemical evidence to explain the pharmacological differences observed between sst_2, sst_3 and sst_5 (SRIF-1) receptors versus sst_1 and sst_4 (SRIF-2) receptors.

2.2.2 Somatostatin Agonists

While both SRIF-14 and SRIF-28 bind readily to all SRIF receptors, sst_5 displays a higher affinity for SRIF-28 (Patel, 1999; Barnett *et al.*, 2003; Olias *et al.*, 2004). Both somatostatin isoforms are rapidly degraded by peptidases (Patel, 1999), which limits their use in experimental designs that involve continuous stimulation for periods of times greater than a few minutes. However, isomerization of the tryptophan residue in SRIF-14 produces [D-Trp^8]-SRIF-14, a peptide analog that binds with high affinity to all receptor subtypes (Patel and Srikant, 1994), and is more resistant to metabolic degradation than the natural ligand (Rivier *et al.*, 1975; Nouel *et al.*, 1997).

Since SRIF-14 and SRIF-28 bind with high affinity to all somatostatin receptor subtypes, neither can be used to study the regulation of individual subtypes in cells or tissues expressing multiple subtypes. While several peptide analogs have been reported to bind with moderate preference for individual receptor subtypes, there is considerable controversy associated with these claims (reviewed in Patel, 1999). However, since the development of several non-peptide subtype-selective agonists by Rohrer and colleagues (1998), the cellular regulation of individual receptor subtypes following agonist binding can be better characterized. For example, L-779,976 has been reported to bind to sst_2 with high affinity and selectivity (Rohrer *et al.*, 1998; Yang *et al.*, 1998), and to potently inhibit intracellular signaling and hormone secretion (e.g. Rohrer *et al.*, 1998; Parmer *et al.*, 1999 Liu *et al.*, 2005).

2.3 Cellular Signaling

2.3.1 G Proteins

The binding of somatostatin to plasma membrane receptors invokes the activation of several effector proteins via pertussis toxin-sensitive and –insensitive heterotrimeric G proteins (Patel, 1999; Csaba and Dournaud, 2001). Upon ligand binding, the receptor undergoes a conformation change that favours the exchange of GDP for GTP on the α-subunit of the associated G protein, which then dissociates from the Gβγ-subunit (Ferguson, 2001). The dissociated Gα- and Gβγ-subunits are in

their active states and can exert negative or positive influences on second messenger proteins. SRIF receptors interact with several possible G proteins, including $G\alpha_{i1-3}$, $G\alpha_{oA}$, $G\alpha_{oB}$, $G\beta_1$, $G\beta_{36}$, $G\gamma_2$ and $G\gamma_3$ (Lahlou *et al.*, 2004). Through these G proteins, SRIF receptors have been shown to regulate several second messenger proteins including adenylate cyclase (AC), Ca^{2+} and K^+ channels, Na^+/H^+ antiporter, guanylate cyclase, phospholipase C, phospholipase A2, mitogen-activated protein kinase, serine/threonine phosphatase and phosphotyrosyl phosphatase (Patel, 1999).

2.3.2 Inhibition of AC

Among the signaling mechanisms that SRIF activates, the negative coupling of $G\alpha_{i1-3}$ to AC, which results in a decrease in cyclic 3', 5'-adenosine monophosphate (cAMP) production, is the most widely studied. Indeed, SRIF has been shown to inhibit cAMP production in several cell types that endogenously express SRIF receptors, including neurons (Grilli *et al.*, 2004), pituitary somatotrophs and pituitary corticotrophs (Csaba and Dournaud, 2001). In the mouse pituitary adenoma cell line AtT-20, SRIF analogs have been demonstrated to inhibit both basal and stimulated cAMP production (e.g. Cervia *et al.*, 2003a; Ben-Shlomo *et al.*, 2005). A decrease in intracellular cAMP levels results in lowered protein kinase A (PKA) activity (Tentler *et al.*, 1997), leading to a decrease in the transcriptional potency of cAMP response element-binding protein

(CREB), and lowered Pit-1/GHF-1 promoter activation. The net result of this sequence of signaling events is a decrease in growth regulatory effects, including the inhibition of hormone secretion (Patel, 1999; Csaba and Dournaud, 2001).

The study of signal transduction pathways in cell lines that endogenously express SRIF receptors is often complicated by the presence of multiple receptor subtypes (Csaba and Dournaud, 2001). Through the exogenous expression of cloned SRIF receptors, however, the functions of individual receptor subtypes can be delineated. For example, in CHO cells expressing cloned sst_2 receptors, stimulation with SRIF has been demonstrated to inhibit cAMP production (e.g. Patel *et al.*, 1994b; Kagimoto *et al.*, 1994; Brasselet *et al.*, 2002; Liu *et al.*, 2005). CHO-K1 cells endogenously express several G proteins that associate with sst_2, including $G\alpha_0$, $G\alpha_{i2}$ and $G\alpha_{i3}$ (Kagimoto *et al.*, 1994; Gu and Schonbrunn, 1997; Sellers *et al.*, 2000). Sst_5 has also been demonstrated to inhibit AC, primarily by coupling to $G_{i/o}$ proteins (e.g. O'Carroll *et al.*, 1992; Patel *et al.*, 1994b; Carruthers *et al.*, 1999). However, at high agonist concentration (> 10^{-7} M), sst_5 may stimulate AC activity by coupling to G α_s (Carruthers *et al.*, 1999).

2.4 Desensitization, Endocytosis and Trafficking

2.4.1 Ligand-Induced Loss of Cell Surface Receptors

Following ligand application, the cell often responds by drastically changing the organization of its GPCRs (Koenig and Edwardson, 1997). Frequently, the result of this receptor re-organization is a decrease in the number of cell surface receptors, concomitant with an increase in the number of intracellular receptors. The loss of cell surface receptors represents a loss of available ligand binding sites, and therefore leads to diminished cellular responsiveness, contributing to a phenomenon called cellular desensitization (Olias *et al.*, 2004). However, cellular desensitization occurs as a result of multiple receptor regulatory mechanisms, and should not be confused with the desensitization of individual GPCRs (which will be covered in the next section). Indeed, under continuous ligand stimulation for periods lasting less than one hour, the net reduction of cell surface receptor density represents a balanced sum of several regulatory events: the desensitization and endocytosis of individual receptors, intracellular trafficking through vesicular compartments and recovery of cell surface receptors through recycling (Koenig and Edwardson, 1997). In addition, longer periods of ligand exposure may lead to receptor up-regulation through the synthesis of new receptors and/or down-regulation of receptors through lysosomal degradation.

2.4.2 A Review of Desensitization, Endocytosis and Trafficking of GPCRs

With respect to individual GPCRs, desensitization can be defined as a loss of functional response due to G protein uncoupling (Perry and Lefkowitz, 2002). However, it must be mentioned that other molecular events, which are beyond the scope of the present discussion, can mediate GPCR desensitization (reviewed in Ferguson, 2001). Receptor desensitization, often referred to as *rapid* desensitization because it occurs within seconds to minutes of ligand exposure, is initiated upon ligand binding and phosphorylation of a cluster of serine/threonine residues in the C-terminal region (Oakley *et al.*, 2001). In this type of desensitization, also referred to as homologous, the receptor is phosphorylated by G protein-coupled receptor kinases (GRKs) or second messenger-activated kinases [e.g. protein kinase C (PKC)] (Ferguson, 2001). In contrast, heterologous desensitization is ligand-independent, occurs through the activation of an independent receptor system and can only take place through second messenger-activated kinases. Phosphorylated receptors are the targets of a family of proteins known as the β-arrestins, which participate in GPCR desensitization by forming a physical barrier between the receptor and the G protein (Perry and Lefkowitz, 2002). This hinders the function of the G protein, and renders the receptor temporarily unresponsive to further stimulation.

The β-arrestins often play a major role in receptor internalization by recruiting clathrin and the AP2 heterotetrameric adapter complex to

facilitate receptor endocytosis via clathrin-coated pits (Laporte *et al.*, 1999; Laporte, 2000). However, some GPCRs internalize completely independent of the β-arrestins, which indicates that this family of proteins is not always essential for internalization (reviewed in Ferguson *et al.*, 2001). Upon recruitment of the required machinery, receptors are sequestered into vesicular compartments, a process that is mediated by the assembly of clathrin subunits (reviewed in Slepnev and De Camilli, 2000). Endocytosis is completed when the vesicle is pinched off by the GTPase dynamin in an energy-dependent final step. Following endocytosis, GPCR-containing vesicles can have several possible fates: they can be targeted to early endosomes, where the receptors are resensitized and sent back to the surface; retained within vesicular compartments or targeted to lysosomes for degradation (Ferguson, 2001).

GPCRs that require β-arrestins to internalize are often categorized into one of two possible groups, Class A and Class B receptors (Perry and Lefkowitz, 2002). Class A receptors, which bind more readily to β-arrestin-2 than β-arrestin-1, separate from the bound β-arrestin during internalization and rapidly recycle back to the cell surface. Class B receptors bind readily to either β-arrestin, remain bound following sequestration and are either detained within endocytotic vesicles, targeted to lysosomes or recycled back to the cell surface in a slow process. Therefore, by mediating the post-endocytotic targeting of receptors, β-arrestins influence receptor fate. However, it must be mentioned that

numerous signaling proteins in the endocytotic pathway help determine the destiny of sequestered receptors (reviewed in McPherson *et al.*, 2001).

Following ligand-induced endocytosis the clathrin coat disassembles, leaving an endocytic vesicle (Ferguson, 2001). Rab GTPases help mediate the subsequent steps of vesicular trafficking, and can be used as markers to determine the identity of different vesicles (Seachrist and Ferguson, 2003). Rab5 assists in the trafficking and fusion of endocytic vesicles with tubulovesicular compartments called early endosomes. Within early endosomes, ligand-receptor complexes dissociate (due to the acidic environment in these compartments), and G protein receptor phosphatases dephosphorylate receptors. Resensitized receptors are then targeted to the plasma membrane for re-insertion. This may occur directly by Rab4 or following trafficking through a separate perinuclear compartment, called the pericentriolar recycling endosome, characterized by its association with Rab11 and Rab4.

2.4.3 Desensitization, Endocytosis and Trafficking of Sst_{2A}

Agonist ligand binding has been shown to promote sst_{2A} phosphorylation, which occurs within minutes of ligand exposure (Hipkin *et al.*, 1997). The overexpression of GRKs increases sst_{2A} phosphorylation in transfected cell lines (Hipkin *et al.*, 1997; Schwartkop *et al.*, 1999; Tulipano *et al.*, 2004) and in pancreatic acinar cells (Elberg *et al.*, 2002). Residues in the 3rd intercellular loop in addition to the C-terminus have

been identified as putative phosphorylation sites (Hipkin *et al.*, 2000). While the evidence for the relationship between sst_{2A} phosphorylation and endocytosis is correlational, there are studies which suggest a link between these two events in cultured somatotrophs (Hipkin *et al.*, 1997; Hipkin *et al.*, 2000) and in human tumours (Liu *et al.*, 2003). Indeed, in a recent study Tulipano *et al.* (2004) reported that internalization and trafficking of sst_{2A} receptors was promoted by over-expression of GRK-2, and that the recruitment of both β-arrestin-1 and –2 is highly dependent on GRK-2-mediated phosphorylation of 3 threonine residues in the C-terminus. However, mutation of the C-terminus of sst_{2A} to ablate GRK phosphorylation sites did not affect the receptor's ability to internalize in HEK-293 cells (Schwartkop *et al.*, 1999), indicating that GRKs may not be essential for sst_{2A} internalization. In hematopoietic cells, sst_2 receptors internalized upon treatment with SRIF, despite the presence of GRK inhibitors (Oomen *et al.* 2001). Furthermore, PKC has been shown to induce sst_{2A} desensitization and potentiate clathrin-mediated endocytosis independent of ligand (Hipkin *et al.*, 2000), and other kinases may also be involved (Hipkin *et al.*, 2000; Ooman *et al.*, 2001). Taken together, these findings suggest that sst_{2A} receptors may undergo homologous or heterologous desensitization and endocytosis following phosphorylation by GRK, PKC or other kinases.

β-Arrestin recruitment to the plasma membrane following stimulation of sst_2 receptors with SRIF-14 has been demonstrated in cells

that endogenously express the receptor (Beaumont *et al.*, 1998; Oomen *et al.*, 2001) and in sst_{2A}-transfected cells (Brasselet *et al.*, 2002; Tulipano *et al.*, 2004; Liu *et al.*, 2005). Using CHO cells co-transfected with mouse sst_{2A} and a dominant negative β-arrestin-1 construct, Brasselet and colleagues (2002) demonstrated that while β-arrestin-1 is required for desensitization, it is not a prerequisite for internalization. Therefore, the precise functions of β-arrestins in the agonist-induced desensitization and endocytosis of sst_{2A} are unclear, and there is growing evidence suggesting that homologous desensitization and endocytosis of sst_{2A} are not directly associated with one another.

Morphological evidence suggests that endogenous SRIF induces internalization of plasma membrane sst_{2A} receptors (Dournaud *et al.*, 1998). Furthermore, there is a convincing body of direct evidence demonstrating that upon stimulation with SRIF-14, human, mouse and rat sst_{2A} receptors undergo rapid endocytosis in a time- and temperature-dependent manner via clathrin-coated pits (e.g. Nouel *et al.*, 1997; Koenig *et al.*, 1998; Boudin *et al.*, 2000; Hipken *et al.*, 2000; Stroh *et al.*, 2000a; Csaba *et al.*, 2001; Brasselet *et al.*, 2002; Tulipano *et al.*, 2004). Using a fluorescent SRIF analog on cultured rat hippocampal neurons and astrocytes, cells that express sst_{2A} endogenously, Stroh and colleagues (2000a) demonstrated that sst_{2A} is found at the cell surface under conditions that inhibit receptor internalization (4°C), and that sst_{2A} receptor-ligand complexes internalized at 37°C. In rat brain slices, it has

been shown that plasma membrane sst_{2A} receptors internalize via endocytic vesicles following stimulation with a SRIF analog (Boudin *et al.*, 2000). Furthermore, endocytosis of sst_{2A} receptors was demonstrated following stereotaxic injection of the sst_{2A} agonist Octreotide® in rats, providing *in vivo* evidence for agonist-induced internalization of plasma membrane sst_{2A} receptors (Csaba *et al.*, 2001).

Following internalization, cell surface sst_{2A} receptors accumulate in the perinuclear region (e.g. Stroh *et al.*, 2000a; Sarret *et al.*, 2004; Tulipano *et al.*, 2004; Liu *et al.*, 2005). In AtT-20 cells, Sarret and colleagues (2004) demonstrated that upon stimulation with SRIF-14, sst_{2A} receptors co-localize in a juxtanuclear compartment immunoreactive for syntaxin-6, a trans-Golgi network (TGN)-pericentriolar recycling endosome marker (Vandenbulcke *et al.*, 2000). Moreover, sequestered sst_{2A} receptors have been shown to dissociate from β-arrestin-2 (Tulipano *et al.*, 2004) and to recycle back to the cell surface (Koenig *et al.*, 1998; Tulipano *et al.*, 2004; Sharif *et al.*, 2005). Taken together, these results suggest that sst_{2A} receptors exhibit characteristics of Class B GPCRs: they bind to β-arrestin-1 or –2, remain bound during internalization and slowly recycle back to the cell surface via the pericentriolar recycling endosome.

2.4.4 Desensitization, Endocytosis and Trafficking of Sst_5

In contrast to the amount of literature on sst_{2A}, relatively little is known about the desensitization, endocytosis and trafficking of sst_5.

Hukovic *et al.* (1998) characterized specific motifs in the C-terminus of the human sst_5 receptor that are involved in SRIF-14-induced desensitization and endocytosis. In CHO cells stably transfected with human sst_5, the receptor internalizes in a time- and temperature-dependent manner following stimulation with SRIF-14 (Hukovic *et al.*, 1996). However, the internalization of sst_5 depends upon the cell type, species of the receptor or type of ligand used (Roth *et al.* 1997; Roosterman *et al.*, 1997; Stroh *et al.* 2000b; Tulipano, 2004). Following stimulation with SRIF-28, the rat sst_5 receptor has been shown to internalize via clathrin coated pits, accumulate in perinuclear vesicles, and recycle back to the cell surface via a pH sensitive process in rat insulinoma cells (Roosterman *et al.*, 1997) and HEK-293 cells transfected with the rat sst_5 receptor (Roth *et al.* 1997). However, no endocytosis was observed following SRIF-14 treatment in either of these cell types. Furthermore, in HEK-293 cells Tulipano *et al.* (2004) demonstrated that while L-817,818 (a sst_5-selective agonist) and SRIF-14 both recruit β-arrestin-2 to the plasma membrane, neither ligand induced endocytosis.

Studies on COS-7 cells transfected with rat sst_5 (Stroh *et al.*, 2000b) and AtT-20 cells (Sarret *et al.*, 1999) demonstrate that cell surface sst_5 immunodensity is maintained following stimulation with SRIF analogs. However, Stroh and colleagues (2000b) showed that while stimulation seemed to induce limited receptor endocytosis as determined by immunocytochemistry, intracellular accumulation of fluorescent and

radioactive ligands was robust. Furthermore, this process was blocked by phenylarsine oxide, an inhibitor of endocytosis, and the recycling inhibitor monensin, suggesting that sst_5 receptors internalize and rapidly recycle back to the cell surface. In the same study, electron microscopy revealed the mobilization of pre-synthesized receptors towards the plasma membrane following stimulation. Taken together, these results suggest that while the sst_5 receptor is internalized upon agonist stimulation, cell surface sst_5 density is maintained or even increased through a combination of receptor recycling and targeting of spare intracellular receptors to the plasma membrane.

3. Somatostatin and the Regulation of GH Release

3.1 The Hypothalamo-Pituitary Axis

SRIF-14 and SRIF-28 are synthesized in neurons of the anterior periventricular nucleus of the hypothalamus (Barnett, 2003). Transcribed from one gene located on the long arm of chromosome 3 in humans, chromosome 16 in mice and chromosome 11 in rats, SRIF messenger ribonucleic acid (mRNA) encodes a 116 amino acid polypeptide called preprosomatostatin (Tannenbaum and Epelbaum, 1999). The first 24 amino acids comprise the signal peptide, which is cleaved upon import into the endoplasmic reticulum to form prosomatostatin. This 92 amino acid proprotein then passes through the endoplasmic reticulum and the Golgi apparatus, undergoing several cleavages to yield SRIF-14 (a tetradecapeptide) and/or SRIF-28 (an octacosapeptide). Following enzymatic cleavage, SRIF-14 and SRIF-28 travel from the anterior hypothalamic periventricular nucleus via axoplasmic transport to the median eminence where they are released next to hypophyseal portal vessels, through which they reach the anterior pituitary gland.

Within the hypothalamus, the most conspicuous SRIF immunoreactivity is found in the anterior periventricular nucleus, but SRIF has also been localized in the suprachiasmatic nucleus, dorso- and ventro-medial nuclei and arcuate nucleus (Finley *et al.*, 1981; Johansson *et al.*, 1984). From the anterior periventricular nucleus, approximately

80% of all somatostatin neurons project to the median eminence (Kawano and Daikoku, 1988). Growth hormone-releasing hormone (GHRH), a peptide produced by neurons in the arcuate nucleus within the hypothalamus, counteracts the inhibitory influence of SRIF by stimulating GH production and secretion (reviewed in Tannenbaum and Epelbaum, 1999). There is also convincing evidence suggesting that SRIF, via fibers projecting to GHRH-producing neurons in the arcuate, inhibits GHRH release directly, and thus regulates GH secretion via central mechanisms (see Fig. 1).

GH secretion from the anterior pituitary gland into the hypophyseal portal blood exhibits an ultradian rhythm (Tannenbaum and Ling, 1984). While there is a basal level of GH secretion that occurs in a pulsatile but irregular pattern throughout the day, bursts of GH release increase in amplitude during REM sleep (Barnett, 2003). GHRH is responsible for the bursts of GH release from the pituitary, while SRIF release regulates the timing and duration (reviewed in Tannenbaum and Epelbaum, 1999; Barnett, 2003). Most of GH's growth-related effects take occur through its induction of insulin-like growth factor (IGF)-I and –II secretion from the liver. Recent findings suggest that SRIF also counteracts the influence of GH peripherally by directly inhibiting IGF-I from the liver (Murray *et al.*, 2004). The GH-IGF-I/II axis is autoregulated by an intricate series of feedback loops, through which the secretion of GH at the level of the pituitary is fine-tuned (Tannenbaum and Epelbaum, 1999) (see Fig.1).

3.2 Sst_2 and Sst_5 Co-operate to Inhibit GH Release from the Pituitary

Early studies demonstrated that binding sites for SRIF exist on several cell types in the anterior pituitary, including somatotrophs (GH releasing cells), thyrotrophs (TSH releasing cells), lactotrophs (PRL releasing cells) and corticotrophs [adrenocorticotropic hormone (ACTH) releasing cells] (reviewed in Tannenbaum and Epelbaum, 1999). However, due to historical developments, the best-defined function of SRIF is its ability to modulate GH release from somatotrophs. The mRNAs for all five receptor subtypes are expressed in the rat pituitary, however sst_2 and sst_5 are the receptor proteins that have been found in highest abundance in somatotrophs (Kumar *et al.*, 1997; Mezey *et al.*, 1998). Furthermore, sst_2 and sst_5 are co-expressed in a proportion of GH-releasing cells (Mezey *et al.*, 1998). Soon after the molecular cloning of individual receptor subtypes, sst_2 was implicated as the subtype that mediates suppression of GH secretion (Raynor *et al.*, 1993). However, studies on human fetal pituitary and pituitary adenoma cells demonstrated that both sst_2 and sst_5 are involved in the regulation of GH release (Shimon *et al.*, 1997a; Shimon *et al.*, 1997b) at the level of the pituitary

Recent evidence raises the interesting possibility of a functional interaction between the sst_2 and sst_5 receptor subtypes in the inhibition of GH secretion at the level of the pituitary gland (Ren *et al.*, 2003; Cervia *et al.*, 2003c). In human fetal pituitary cultures, the use of a combination of sst_2- and sst_5-preferring or sst_2/sst_5 biselective SRIF analogs induced

more GH suppression than did treatment with either sst_2 or sst_5-preferring analogs alone, suggesting that sst_2 and sst_5 act synergistically to modulate GH release (Ren *et al.*, 2003). Moreover, this effect was reversed by co-treatment with a sst_2-selective antagonist, indicating that the co-stimulation of sst_2 and sst_5 is required for the inhibition of GH release. Evidence from a study on GC rat somatotroph cells suggests that sst_5 may modulate sst_2-induced cAMP inhibition (Cervia *et al.*, 2003c). In addition, a recent study by Ben-Shlomo and colleagues (2005) demonstrated that sst_5 regulates sst_2 signaling in AtT-20 cells. Taken together, these studies suggest that the inhibition of GH release is mediated through a functional interaction between sst_2 and sst_5 receptors, however the exact mechanism of this interaction is unknown.

3.3 Acromegaly: a Disorder of GH Release

3.3.1 Etiology and Clinical Description

In 95% of all cases, acromegaly, a syndrome that is characterized by GH hypersecretion, is caused by a benign pituitary adenoma (Heaney and Melmed, 2004; Muller and van der Lely, 2004). The classical clinical signs of acromegaly are associated with hypersomatotropism, which is characterized by enlarged body extremities and joint disfigurement, hypertension, cardiovascular disease, cerebrovascular disease and diabetes mellitus. Surgical treatment to remove pituitary adenomas has variable success, and there is significantly high post-operative morbidity

associated with the procedure (reviewed in Heaney and Melmed, 2004). The majority of pituitary adenomas occur sporadically and little is known about their etiology and development. While few heritable abnormalities have been associated with the pituitary adenomas, it is estimated that in 40% of all cases pathological hormone hypersecretion from the tumour results from point mutations in the region encoding the GTP binding domain of $G\alpha_s$ proteins. This results in constitutive adenylate cyclase activity, which leads to a marked increase in the amplitude of GH bursts and the level of baseline GH secretion (Racine and Barkan, 2003).

3.3.2 Pharmacological Therapies

Greater than 90% of all pituitary adenomas express sst_2 or sst_5 receptors (Heaney and Melmed, 2004). This expression pattern coupled with the evidence that sst_2 and sst_5 co-operate in the release of GH makes sst_2 and sst_5 ideal targets for pharmacological treatments. Since somatostatin has a half-life of about two minutes in the body, the native peptide is not suitable for use as a pharmacological agent. However, several stable SRIF analogs have been developed over the last 20 years for the treatment of acromegaly (Racine and Barkan, 2003). Octreotide® and Lanreotide®, which bind about 10 times better to sst_2 than sst_5, are the most frequently prescribed SRIF analogs (Hofland and Lamberts, 2003).

It is estimated that up to two thirds of all cases of acromegaly can be treated successfully with Octreotide®, which has a half-life of about 2 hrs in the body and is 45 times more potent than somatostatin (Kleinberg, 2005). Several multi-centered trials have demonstrated that this drug significantly lowers GH levels, and in turn dramatically reduces IGF-I release (Heaney and Melmed, 2004). Injected 3 times per day or implanted as a subcutaneous slow-release capsule, Octreotide® treatment takes effect within hours to days in 75% of all patients (Newman, 1995). In addition to normalizing GH secretion, another therapeutic benefit of Octreotide® is its influence on shrinking tumour mass (Hofland and Lamberts, 2003; Heaney and Melmed, 2004). The side effects associated with Octreotide® treatment, including gastro-intestinal problems and hyperglycemia, usually disappear within 3 months due to downregulation of SRIF receptors in peripheral tissues (Heaney and Melmed, 2004).

However, approximately one third of all acromegaly patients do not respond to Octreotide® or Lanreotide® (Kleinberg, 2005). Over the last few years, several experimental pharmacological agents have been under development with the goal of providing treatment options for these patients. In pituitary adenomas collected from five patients who were only partially responsive to Octreotide®, Saveanu and colleagues (2001) demonstrated that a sst_2/sst_5 biselective SRIF peptide analog provided superior GH inhibition. SOM-230, an experimental SRIF peptide analog that has not yet been approved for clinical use, binds readily to all the

SRIF receptor subtypes except sst_4, but binds to sst_2 and sst_5 with the highest affinity (Bruns *et al.*, 2002; Weckbecker *et al.*, 2002). Interestingly, this compound has been demonstrated to be more potent and last longer than Octreotide®, and have a profound effect on decreasing IGF-1 levels in experimental animals and humans (Kleinberg, 2005). In summary, pharmacological evidence suggests that co-stimulation of sst_2 and sst_5 provides superior inhibition of GH release from pituitary adenomas.

3.3.3 The Development of Drug Tolerance

Typically associated with normal hormone secretion, adaptation or tachyphylaxis develops after continuous receptor stimulation within hours to days (Patel, 1999; Tannenbaum and Epelbaum, 1999). With respect to pharmacological therapy, tachyphylaxis or tolerance necessitates the use of increasing doses of a drug to receive the same desired outcome. Interestingly, GH-releasing pituitary adenomas are treated very successfully with SRIF analogs without promoting the development of drug tolerance (Hofland and Lamberts, 2003). This is in stark contrast to other common treatments targeting neuropeptide receptors such as opiates in pain therapy, which are commonly plagued by the development of tolerance.

A comparison between Octreotide® therapy for pituitary adenomas versus islet-cell tumours and carcinoids reveals remarkable differences with respect to the development of tachyphylaxis (Hofland and Lamberts,

2003). As is the case for pituitary adenomas, Octreotide® is often prescribed to treat islet-cell tumours and carcinoids with rapid improvement of symptoms. However, unlike patients with pituitary adenomas, those with islet-cell tumours and carcinoids develop desensitization to Octreotide® within weeks to months of initial treatment (Lamberts *et al.*, 1996). Although increasing the dosage of the drug may initially reverse tolerance, the patient eventually becomes non-responsive to Octreotide®. In the acromegaly literature, reports of desensitization to Octreotide® treatment are virtually absent, and patients receive excellent results using the same dosage of the drug for several years (Hofland and Lamberts, 2003). This indicates that the mechanisms involved in maintenance of drug responsiveness are likely due to intrinsic properties of pituitary adenomas.

4. Rationale of the Present Master's Thesis

Although patients with pituitary adenomas are often successfully treated with SRIF analogs that target sst_2 and sst_5 receptors without the development of tolerance, the underlying cellular processes that mediate this clinical phenomenon are unknown (Hofland and Lamberts, 2003). The development of tolerance to pharmacological agents targeting other G protein-coupled neuropeptide receptors such as the opioid receptors are thought to be influenced by agonist-induced changes in receptor trafficking (Reviewed in von Zastrow, 2004). However, it is not clear if the absence of tolerance to SRIF analogs in the treatment of acromegaly is also mediated by changes in SRIF receptor regulation. Therefore, the overall objective of the present Master's thesis was to investigate the mechanisms whereby cells expressing SRIF receptors remain responsive to SRIF analogs under continuous stimulation.

Previously, our laboratory demonstrated that sst_{2A} (the most prevalent sst_2 gene transcript) and sst_5 exhibit very different modes of regulation following stimulation with [D-Trp^8]-SRIF-14. Whereas sst_{2A} is removed very rapidly from the cell surface through endocytosis (Sarret *et al.*, 1999; Stroh *et al.*, 2000a; Brasselet *et al.*, 2002), cell-surface sst_5 receptors are maintained in the presence of high agonist levels (Sarret et al., 1999; Stroh *et al.*, 2000b) through a combination of rapid recycling and membrane targeting of spare receptors (Stroh *et al.*, 2000b). However, since [D-Trp^8]-SRIF-14 binds with high affinity to all of its receptor

subtypes (Patel and Srikant, 1994), the independent regulation of sst_{2A} and sst_5 in cells expressing multiple receptor subtypes could not be ascertained in these previous studies.

Recent studies examining sst_2- and sst_5-mediated actions of SRIF-14 indicate that a functional interaction between these receptor subtypes influences the inhibition of hormone release, however the precise mechanisms mediating this effect are unknown (Cervia *et al.*, 2003c; Ren *at al.*, 2003; Ben-Shlomo *et al.*, 2005). The characterization of the hypothesized sst_2-sst_5 interaction has been challenging because most SRIF analogs bind to sst_2 and sst_5 with similar affinities (Patel, 1999). Therefore, the individual contribution of each receptor to this interaction cannot be delineated using these analogs. As a result, while there have been several studies examining the agonist-induced trafficking of sst_2 and sst_5 in cells expressing either receptor alone, the regulatory events associated with the co-expression of sst_2 and sst_5 have not been fully investigated.

The primary objective of the present Master's thesis was to characterize the cellular regulation of sst_{2A} and sst_5 receptors in cells expressing both receptor subtypes, so that we could better understanding the cellular mechanisms involved in the maintenance of responsiveness to SRIF analogs. We hypothesized that the presence of sst_5 affects the agonist-induced internalization, intracellular trafficking and signaling of sst_{2A} receptors. To test our hypothesis, we first established the regulatory

events associated with each receptor subtype independently using epithelial cell lines that ectopically expressed either sst_{2A} or sst_5 alone. This was followed by detailed analysis of the regulation of sst_{2A} receptors using a highly selective sst_2 agonist in an epithelial cell line that co-expressed sst_5 ectopically. Finally, we studied the effect of sst_5 expression on sst_{2A} regulation in a pituitary adenoma cell line endogenously expressing sst_{2A} and sst_5 to delineate the mechanisms that mediate the maintenance of responsiveness to SRIF analogs in a clinically relevant cell model. Therefore, by understanding the regulatory processes that mediate the absence of tolerance to SRIF analogs in acromegaly patients, we may be able to provide more general insight into the basic cellular mechanisms underlying the development, or absence, of tolerance to drugs acting at GPCRs.

Materials and Methods

1. Plasmid construction

To construct N-terminally c-Myc-tagged sst_{2A} and HA-tagged sst_5 receptors, mouse sst_{2A} and sst_5 cDNAs were amplified from plasmid pCMV-6b by polymerase chain reaction using 5'- and 3'-oligonucleotide primers containing NotI-BamHI and EcoRI-NotI restriction site sequences, respectively. PCR products were gel-purified, digested and directly inserted between the corresponding sites of plasmids pIRES-neo (cMyc-sst_{2A}) and pIRES-puro (HA-sst_5) (Clontech; Palo Alto, CA). Constructs were verified by sequencing.

2. Culture and transfection of cells

CHO-K1 (American Type Culture Collection; Rockville, MD) were maintained in Ham's F12 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin/streptomycin and 250 μg/ml Fungizone (Invitrogen, Burlington, ON, Canada). To establish stable cell lines expressing c-Myc-sst_{2A} and/or HA-sst_5 receptors, 2.6 x 10^6 CHO-K1 cells were transfected with 8 μg of c-Myc-sst_{2A} pIRES-neo or HA-sst_5 pIRES-puro plasmids, using a Lipofectamine transfection reagent (DAC30, Eurogentec, San Diego, CA) according to the manufacturer's instructions. Individual clones of transfected cells were selected in the presence of 750

μg/ml geneticin G418 (Invitrogen) or 25 μg/ml puromycin (BD Clonetech, Mountain View, CA), respectively, and tested for their capacity to bind $^{125}Tyr^{0}$-D-Trp^{8}-SRIF-14 in a radioligand binding assay. A clone expressing 600 fmol/mg of sst_{2A} receptor (CHO-sst_{2A}) was used to generate CHO-K1 cells stably expressing both receptor subtypes (CHO-sst_{2A+5}) using the same protocol.

Mouse AtT-20/D16-16 tumor cells were grown in Dulbeco's modified Eagle's medium (DMEM) with high glucose supplemented with 10% FBS, 10 % horse serum, 100 units/ml penicillin/streptomycin and 250 μg/ml Fungizone. All cells were cultured in 100 mm dishes, maintained in a humidified atmosphere of 95% air and 5% CO_2, and passed when the monolayer reached 90% confluence.

3. Competition binding of [D-Trp^{8}]-SRIF-14 or L-779,976 using ^{125}I-Tyr^{0}[D-Trp^{8}]-SRIF-14

To determine the binding affinities of [D-Trp^{8}]-SRIF-14, a non-subtype selective somatostatin peptide analog that is more resistant to metabolic degradation than native SRIF-14 and is 6-8 time more potent than the natural ligand (Rivier *et al.*, 1975; Reubi *et al.*, 1982; Nouel *et al.*, 1997), and L-779,976, a non-peptidic somatostatin analog that shows high affinity and selectivity for sst_{2A} (Rohrer *et al.*, 1998; Yang *et al.*, 1998), competition binding experiments were performed on whole live CHO-sst_{2A}, CHO-sst_{5}, CHO-sst_{2A+5} and AtT-20 cells. Cells were washed once and

equilibrated for 10 min at 37°C with Earle's buffer (140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.9 mM $MgCl_2$ and 25 mM Hepes), pH 7.4, containing 2% BSA. Next, 0.3 nM of ^{125}I-Tyr^0[D-Trp^8]-SRIF-14 (1800-2000 Ci/mmol; J. Mazella, Valbonne, France) was added to the equilibrium mixture in the presence of increasing concentrations (from 10^{-12} to 10^{-6} M) of either unlabeled [D-Trp^8]-SRIF-14 or L-779,976 for 30 min. Nonspecific binding was measured in the presence of 1 µM of unlabeled [D-Trp^8]-SRIF-14. Subsequently, cells were washed twice with Earle's buffer and detached using 0.1 M NaOH. The radioactivity of each sample was counted in a γ-counter. Competition binding experiments were done in triplicate and repeated twice. IC_{50} values were calculated using nonlinear regression analysis in SigmaPlot version 7.0 (SPSS Inc., Point Richmond, CA).

4. ^{125}I-Tyr^0[D-Trp^8]-SRIF-14 cell surface binding

Radioactive ligand binding was performed on live CHO-sst_{2A}, CHO-sst_{2A+5} and AtT-20 cells at 37°C to quantify cell surface binding sites following stimulation with [D-Trp^8]-SRIF-14 or L-779,976. At least 12 hrs before each experiment, approximately 300,000 cells per well were plated on 24 well tissue culture plates (Falcon, Franklin Lakes, NJ). Before stimulation, cells were equilibrated for 10 min at 37°C in Earle's buffer, pH 7.4, containing 2% BSA and 0.1% D-glucose. The equilibrium mixture was replaced by 300 µl of Earle's buffer containing 0.8 mM 1,10-phenanthroline and 100 nM of either [D-Trp^8]-SRIF-14, L-779,976 or

Earle's buffer alone for 0-40 min. Following agonist stimulation, cells were washed three times with ice cold Earle's buffer, twice with a hypertonic acid solution (Earle's buffer containing 0.2 M acetic acid and 0.5 M NaCl, pH 4) to wash away surface-bound ligand, and three times again with Earle's buffer. Next, to quantify the remaining surface binding sites, cells were incubated with 250 µl of Earle's buffer containing 2% BSA, 0.3 nM of ^{125}I-Tyr^{0}[D-Trp^{8}]-SRIF-14 (1800-2000 Ci/mmol; Phoenix Pharmaceuticals, Belmont, CA) for 30 min at 37°C. To inhibit receptor internalization during radioactive labeling, 10 µM of phenylarsine oxide (PAO; a well-documented internalization blocker, Hertel *et al.*, 1985; for a review see Koenig and Edwardson, 1997) was added to the above mixture. We repeated the competition binding experiments described earlier in the presence of PAO to determine whether this compound could affect binding affinity. In addition, to verify that PAO blocked receptor internalization, we repeated the present experiments in the absence of PAO, and incubated cells with ^{125}I-Tyr^{0}[D-Trp^{8}]-SRIF-14 at 4°C. Nonspecific binding was measured in the presence of 10 µM of unlabeled [D-Trp^{8}]-SRIF-14, and represented less than 10% of the total binding. Total specific surface binding in stimulated cells was normalized to the total specific binding in unstimulated cells (100%). In CHO cells, the data for five separate experiments (each performed in triplicate) was pooled, and statistical significance was verified using a One-way ANOVA followed by Bonferroni's Multiple Comparison Test. In AtT-20 cells, the data for four

separate experiments (each performed in triplicate) were pooled, and statistical significance was verified using an unpaired t-test with Welch's correction.

5. Sst_{2A} and sst_5 immunocytochemistry

To characterize the trafficking of sst_{2A} and sst_5 following stimulation with 100 nM [D-Trp^8]-SRIF-14 or L-779,976, we conducted immunocytochemical analysis on CHO-sst_{2A}, CHO-sst_5, CHO-sst_{2A+5} and AtT-20 cells. Approximately 100,000 cells per well were plated on poly-L-lysine coated glass coverslips in four-well tissue culture plates (Nunc, Roskilde, Denmark) at least 12 hrs before each experiment. Following agonist stimulation for 0-40 min (as described above), cells were fixed with 4% paraformaldehyde (Polysciences, Warrington, PA) for 20 min and pre-incubated with a blocking solution for 15 min containing 5% NGS, 2% BSA and 0.1% Triton X-100 in Tris Buffered Saline (TBS). Immunostaining was performed by incubating cells overnight at 4°C in TBS containing 0.05% Triton X-100, 1% Normal Goat Serum (NGS; Jackson ImmunoResearch, West Grove, PA) and primary antibody. In CHO cells, c-Myc-sst_{2A} was detected using a rabbit antibody (1:500; Sigma, St. Louis, MO) directed against the c-Myc epitope tag, while HA-sst_5 was detected using a mouse antibody (1:500; Roche, Indianapolis, IN) directed against the HA epitope tag. Untransfected CHO-K1 cells were used as a control to ensure antibody specificity.

In AtT-20 cells, sst_{2A} and sst_5 were detected using rabbit antibodies (1:500; Gramsch Laboratories, Schwabhausen, Germany) directed against the C-terminal segments of the mouse sst_{2A} and sst_5 receptors. Bound antibodies were revealed using goat anti-rabbit Alexa 488-, and/or goat anti-mouse Alexa 594-conjugated secondary antibodies (1:500; Molecular Probes, Eugene, OR). Immunocytochemistry experiments were all performed in duplicate and repeated at least three times.

Immunolabeled cells were observed on a Zeiss Laser Scanning Microscope (LSM) 510 equipped with Argon2 (488 nm) and He/Ne1 (543 nm) lasers. Single-labeled cells were analyzed using either laser to excite fluorophores, while images of double-labeled cells were acquired by using both lasers simultaneously in *Multitrack* mode. Images were processed with the Zeiss LSM 510 software and Photoshop 6.0 (Adobe).

6. Quantification of cell surface sst_{2A} immunofluorescence

We quantified cell surface c-Myc-sst_{2A} following 40 min of stimulation with 100 nM [D-Trp^8]-SRIF-14 or L-779,976 to characterize the agonist-induced loss of cell surface sst_{2A} receptor density in CHO-sst_{2A} and CHO-sst_{2A+5} cells. Approximately 300,000 cells per well were plated on 24 well tissue culture plates (Falcon) at least 12 hrs before each experiment. Following 40 min of agonist stimulation (as described above), cells were rinsed with ice-cold Earle's buffer and incubated with a solution containing rabbit anti-c-Myc (1:500; Sigma), 2% BSA and 1% NGS for 90

min at 4 °C (to inhibit receptor endocytosis). They were then fixed with 4% PFA for 20 min at RT, incubated for 1 hr with a goat anti-rabbit Alexa 488-conjugated secondary antibody (1:500; Molecular Probes), and fluorescence intensity was measured on a FL600 fluorescence plate reader (Fischer Scientific, Montreal, QC, Canada). Means for each condition were calculated and subtracted from background fluorescence (wells not treated with primary antibody). Surface fluorescence intensity in stimulated cells was normalized to fluorescence intensity in unstimulated cells (100%). The data for three separate experiments (each done in quadruplicate) was pooled and statistical significance was verified using a One-way ANOVA followed by Bonferroni's Multiple Comparison Test.

Since the only commercially available antibodies for sst_{2A} receptors are directed at the C-terminus, immunocytochemical detection of endogenously expressed sst_{2A} receptors requires permeablization. Therefore, we analyzed our confocal images to quantify peripheral sst_{2A} immunofluorescence in AtT-20 cells. Images were converted to grayscale using Photoshop 6.0 (Adobe) and imported into ImageJ (http://rsb.info.nih.gov/ij/) for grey level intensity analysis. Five images for each condition were used for analysis and each was divided into quadrants. Four to six cells from each quadrant were selected at random; the peripheral fluorescence traced by hand and the mean grey level of each traced area was calculated. Peripheral fluorescence intensity of stimulated cells was normalized to the peripheral fluorescence intensity of

unstimulated cells (100%). The data for three separate experiments (each performed in duplicate) were pooled, and statistical significance was verified using a One-way ANOVA followed by Bonferroni's Multiple Comparison Test.

To investigate whether the re-appearance of surface sst_{2A} involved pH-dependent sorting in endosomes in CHO-sst_{2A} and CHO-sst2A+5 cells, we used monensin, an ionophore that has been demonstrated to prevent SRIF receptor recycling (Green and Shields, 1984; Roosterman *et al.*, 1997; Beaumont *et al.*, 1998). Approximately 300,000 cells per well were plated on 24 well tissue culture plates (Falcon) at least 12 hrs before each experiment. Fifteen minutes before the start of the experiment, medium was replaced or not by F12 medium containing 25 μM of monensin. Cells were equilibrated at 37 °C for 10 min in either Earle's buffer containing 2% BSA and 1% glucose or Earle's buffer supplemented with 25 μM of monensin followed by 40 min of stimulation with 100 nM of either [D-Trp^8]-SRIF-14 or L-779,976 with or without 25 μM monensin in Earle's buffer, or Earle's buffer alone with or without 25 μM monensin. Subsequent to a hypertonic acid wash, to wash away surface-bound ligand, cells were incubated at 37 °C in the absence of ligand for 0-40 min in Earle's buffer with or without 25 μM of monensin, to allow for re-appearance of receptors at the cell surface. Following the recovery period, cell surface sst_{2A} immunofluorescence was quantified using the fluorescence plate reader as described above. Surface fluorescence

intensity in stimulated cells was normalized to fluorescence intensity in unstimulated cells (100%). The data for three separate experiments (each done in quadruplicate) was pooled and statistical significance was verified using a One-way ANOVA followed by Bonferroni's Multiple Comparison Test.

7. cAMP assay

To investigate the responsiveness of CHO-sst_{2A}, CHO-sst_{2A+5} and AtT-20 cells following pre-stimulation with [D-Trp^8]-SRIF-14 or L-779,976, we measured their ability to inhibit forskolin-stimulated cAMP production. Approximately 200,000 cells per well were plated on 24 well tissue culture plates (Falcon) at least 12 hrs before each experiment. Cells were equilibrated and pre-stimulated with 100 nM of [D-Trp^8]-SRIF-14 or L-779,976 for 40 min. This was followed by a second stimulation with either 100 nM of forskolin (Sigma) and 1 mM IBMX (Sigma) or a mixture containing 100 nM of [D-Trp^8]-SRIF-14 or L-779,976, 100 nM forskolin and 1mM IBMX. The experiment was terminated by adding lysis reagent (0.5% dodecyltrimethylammonium bromide) to each well for 20 min. Total adenylate cyclase inhibition was measured with an enzyme immunoassay (EIA) kit, according to the manufacturer's suggestions (Amersham Biosciences, Buckinghamshire, UK). Cellular extracts were transferred to a 96-well plate (pre-coated with donkey anti-rabbit IgG), and rabbit antiserum against cAMP was added to each well at 4°C for 2 hrs. Next,

cAMP conjugated to horseradish peroxidase was added for 60 min to initiate competition, which was terminated by extensive washes with 0.01 M phosphate buffer, pH 7.4, containing 0.05% (v/v) Tween 20 (EMD Chemicals, Gibbstown, NJ). Enzyme substrate were added to each well where a blue color was allowed to develop for 1 hr; the plate was then read at 630 nm on a μQuant microplate reader (Bio-Tek Instruments Inc, Winooski, VT). Cells receiving no pre-treatment followed by forskolin alone were used to calculate maximum cAMP production. The effect of agonist on forskolin-stimulated cAMP production was expressed as a percentage of the effect of forskolin alone (forskolin effect). The data for four separate experiments (each done in triplicate) was pooled and statistical significance was verified using a One-way ANOVA followed by Tukey's Multiple Comparison Test.

Results

1. Binding affinities of [D-Trp^8]-SRIF-14 and L-779,976

To determine the binding affinities of [D-Trp^8]-SRIF-14 and L-779-976 in whole live CHO-K1 cells stably expressing mouse sst_{2A} (CHO-sst_{2A}), sst_5 (CHO-sst_5), both receptor constructs (CHO-sst_{2A+5}), and in cells endogenously expressing sst_{2A} and sst_5 (AtT-20 cells), we performed competition binding experiments using ^{125}I-Tyr^0[D-Trp^8]-SRIF-14 at 37°C and pH 7.4. [D-Trp^8]-SRIF-14 bound to sst_{2A} with high affinity in CHO-sst_{2A} with an IC_{50} of 0.54 nM, and bound to cells simultaneously expressing sst_{2A} and sst_5 either exogenously (CHO-sst_{2A+5}) or endogenously (AtT-20 cells) with affinities in the same range (Table 1). While L-779,976 bound readily to sst_{2A} in all cell types, it displayed a lower affinity for sst_{2A} than did [D-Trp^8]-SRIF-14. Nevertheless, L-779,976 did not bind to sst_5 in CHO-sst_5, confirming that this agonist was selective for sst_{2A} over sst_5 for concentrations up to 100 nM. Therefore, we worked at concentrations of 100 nM for both [D-Trp^8]-SRIF-14 and L-779,976 in the subsequent experiments. In addition, we determined that PAO had no appreciable effect on binding affinities for either ligand (data not shown).

2. ^{125}I-Tyr^0[D-Trp^8]-SRIF-14 cell surface binding to CHO-sst$_{2A}$, CHO-sst$_{2A+5}$ and AtT-20 cells

To quantify the remaining cell surface binding sites following 0-40 min of stimulation with [D-Trp^8]-SRIF-14 or L-779,976, cells were first acid washed to strip off surface-bound ligand. This was followed by radioactive ligand binding using ^{125}I-Tyr^0[D-Trp^8]-SRIF-14 under conditions that inhibit receptor internalization (i.e. in the presence of PAO). Upon stimulation of CHO-sst$_{2A}$ cells (Fig. 2*A*) with either [D-Trp^8]-SRIF-14 (□) or L-779,976 (○), there was a time-dependent decrease in ^{125}I-Tyr^0[D-Trp^8]-SRIF-14 surface binding, which suggests that both agonists induced endocytosis of cell surface sst$_{2A}$ receptors efficaciously. Cell surface binding leveled off after 10 min and was maintained up to 40 min, after which there was approximately 20 and 25% remaining surface binding sites for cells stimulated with [D-Trp^8]-SRIF-14 and L-779,976, respectively.

In CHO-sst$_{2A+5}$ cells (Fig. 2*A*), stimulation with [D-Trp^8]-SRIF-14 (■) resulted in approximately the same decrease in surface binding as was observed in CHO-sst$_{2A}$ cells (□). In contrast, whereas stimulation of CHO-sst$_{2A+5}$ cells with L-779,976 (●) also induced a time-dependent decrease in ^{125}I-Tyr^0[D-Trp^8]-SRIF-14 binding, this effect was considerably reduced compared with [D-Trp^8]-SRIF-14 stimulation in the same cells (○; $p \leq 0.01$; $n = 5$), resulting in approximately 60% remaining cell surface receptors after 40 min of stimulation. Furthermore, the decrease in cell surface binding sites following stimulation with L-779,976 was also reduced as

compared to stimulation with the same agonist in CHO-sst_{2A} cells ($p \leq 0.01$, $p \leq 0.001$; $n = 5$). Incubation of cells with ^{125}I-Tyr^0[D-Trp^8]-SRIF-14 at 4°C in the absence of PAO (data not shown) yielded very similar binding data, confirming that PAO effectively inhibited internalization.

Binding experiments in AtT-20 cells (Fig. 2*B*) revealed a pattern that resembled our findings in CHO-sst_{2A+5} cells. Following stimulation with [D-Trp^8]-SRIF-14 (▽), there was a time-dependent decrease of surface ^{125}I-Tyr^0[D-Trp^8]-SRIF-14 binding, which leveled off by 10 min. After 40 min of [D-Trp^8]-SRIF-14, there was approximately 40% remaining cell surface binding sites. However, following L-779,976 treatment (▼), surface binding sites were maintained within the first 10 min of stimulation. This was followed by a decrease in surface binding that reached equilibrium after 20 min, and resulted in approximately 75% remaining cell surface binding sites after 40 min of stimulation, significantly more than follwing [D-Trp^8]-SRIF-14 stimulation ($p \leq 0.01$, $p \leq 0.001$; $n = 4$).

3. Trafficking of c-Myc-sst_{2A} in CHO-sst_{2A} and CHO-sst_{2A+5} cells

To characterize the agonist-induced internalization and trafficking of sst_{2A} in cells exogenously expressing sst_{2A} (CHO-sst_{2A}) or both sst_{2A} and sst_5 (CHO-sst_{2A+5}), we conducted immunocytochemical analysis using an antibody directed against the c-Myc epitope tag (Fig. 3). Confocal imaging revealed that in the absence of ligand, anti-c-Myc-sst_{2A} immunostaining was localized at the cell periphery in CHO-sst_{2A} (Fig. 3*A* and *B*) and in CHO-sst_{2A+5} cells (Fig. 3*I* and *J*). After 5 min of stimulation with [D-Trp^8]-SRIF-14 (Fig. 3*C* and *K*), sst_{2A} immunostaining was lost from the cell periphery, and immunofluorescent puncti resembling endosomes began to accumulate in the cytoplasm. After 20 and 40 min, this fluorescence accumulated in the perinuclear region in both cell types (Fig. 3, *E, G, M, O*). In CHO-sst_{2A} cells, stimulation with L-779,976 (Fig. 3*D, F, H*) induced similar sst_{2A} internalization and trafficking events as those observed following [D-Trp^8]-SRIF-14 stimulation.

Following stimulation with L-779,976 in CHO-sst_{2A+5} cells, sst_{2A} immunofluorescence was also mobilized to punctiform compartments, however these remained near the cell periphery (Fig. 3*L, N*). Following 40 min of stimulation with L-779,976 (Fig. 3*P*), peripheral immunofluorescence recovered, and there was only very limited perinuclear accumulation compared to [D-Trp^8]-SRIF-14 stimulation in the same cells (Fig. 3*O*), and to stimulation with L-779,976 in CHO-sst_{2A} cells (Fig. 3*H*).

To compare cell surface c-Myc-sst_{2A} receptor density before and after 40 min of stimulation with [D-Trp^8]-SRIF-14 or L-779,976, we immunostained CHO-sst_{2A} and CHO-sst_{2A+5} cells at 4°C (to inhibit receptor internalization) using an antibody directed against the N-terminal c-Myc epitope tag. Cell surface immunofluorescence was subsequently assessed by confocal microscopy or measured using a fluorescence plate reader (Fig. 4). In the absence of ligand, intense c-Myc-sst_{2A} immunostaining was observed by confocal microscopy at the cell surface in CHO-sst_{2A} (Fig. 4*A*) and CHO-sst_{2A+5} cells (Fig. 4*D*). In comparison, following stimulation with [D-Trp^8]-SRIF-14, there was little cell surface sst_{2A} left in both cell types (Fig. 4*B and E*), resulting in approximately 25% remaining surface immunofluorescence intensity (Fig. 4*G, grey bars*).

Stimulation of CHO-sst_{2A} cells with L-779,976 (Fig. 4*C*) resulted in a very similar decrease in cell surface sst_{2A} as was observed following [D-Trp^8]-SRIF-14 stimulation, resulting in 30% remaining cell surface immunofluorescence (Fig. 4*G, black bar*). However, following stimulation of CHO-sst_{2A+5} cells with L-779,976 (Fig. 4*F*), more cell surface sst_{2A} immunofluorescence remained. L-779,976 stimulation resulted in approximately 45% remaining cell surface immunofluorescence (Fig. 4*G, black bar*), which was significantly greater than what remained after [D-Trp^8]-SRIF-14 stimulation in the same cells ($p \leq 0.001$; $n = 3$) and after L-779,976 stimulation in CHO-sst_{2A} cells ($p \leq 0.05$; $n = 3$).

To characterize the re-appearance of cell surface c-Myc-sst_{2A} receptors following 40 min of agonist stimulation at 37°C and pH 7.4, we performed a hypertonic acid wash to strip off surface-bound ligand. Following a recovery period in the absence of ligand, we quantified cell surface immunofluorescence as described above. In CHO-sst_{2A} cells (Fig. 5*A* and *B*), surface sst_{2A} re-appearance occurred in a time-dependent manner. This re-appearance was independent of whether the cells were stimulated with [D-Trp^8]-SRIF-14 (Fig. 5*A, black bars*) or with L-779,976 (Fig. 5*B, black bars*). Furthermore, the re-appearance of cell surface sst_{2A} receptors was inhibited by monensin (Fig. 5*A, B, grey bars*; $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$; $n = 3$), indicating that recycling of sst_{2A} receptors occurred through a pH-dependent process. In CHO-sst_{2A+5} cells, the pattern of cell surface sst_{2A} re-appearance observed after stimulation with [D-Trp^8]-SRIF-14 (Fig. 5*C, black bars*) was similar to that observed in cells expressing sst_{2A} alone, and was inhibited by monensin (Fig. 5*C, grey bars*; $p \leq 0.05$, $p \leq 0.01$; $n = 3$). However, in CHO-sst_{2A+5} cells that were stimulated with L-779,976 (Fig. 5*D, black bars*), there was no re-appearance of cell surface sst_{2A} and monensin had no effect (Fig. 5*D, grey bars*), suggesting that recycling did not occur.

4. Trafficking of sst_{2A} in AtT-20 cells

To characterize the internalization and trafficking of sst_{2A} in AtT-20, a cell line that endogenously expresses sst_{2A} and sst_5, we conducted immunocytochemical analysis (Fig. 6). Confocal imaging revealed that in the absence of ligand, sst_{2A} immunostaining was pronounced at the cell periphery (Fig. 6*A and B*). Upon stimulation with [D-Trp^8]-SRIF-14 Fig. 6*C, E, G*), there was rapid internalization and perinuclear accumulation of sst_{2A} immunofluorescence. Following 40 min of stimulation with [D-Trp^8]-SRIF-14 (Fig. 6*G*), peripheral sst_{2A} staining was limited, and pronounced immunopositive clusters were present in the perinuclear region. In contrast, stimulation with L-779,976 (Fig. 6*D, F, H*) resulted in enduring sst_{2A} immunolabeling at the cell periphery, limited appearance of sst_{2A}-positive puncti in the cytoplasm and little perinuclear accumulation of immunoreactive particles after 40 min (Fig. 6*H*).

Since the available antibodies specific for sst_{2A} are all directed against its C-terminus, immunocytochemical detection of endogenously expressed sst_{2A} receptors requires permeablization. Therefore, we quantified sst_{2A} density at the cell periphery following stimulation with [D-Trp^8]-SRIF-14 or L-779,976 by conducting grey level analysis of peripheral sst_{2A} immunofluorescence in confocal images. Following stimulation with either ligand, peripheral sst_{2A} immunolabeling decreased with time and reached equilibrium after 20 min (Fig. 6*I*). After 40 min of stimulation with [D-Trp^8]-SRIF-14 (Fig. 6*I, grey bars*), there was approximately 35%

remaining peripheral sst_{2A} immunofluorescence. However, in cells stimulated with L-779,976 for 40 (Fig. *6I, black bars*), there was approximately 55% remaining peripheral sst_{2A} immunofluorescence, suggesting that cell surface sst_{2A} receptor density was maintained as compared with [D-Trp^8]-SRIF-14-stimulated cells ($p \leq 0.001$; $n = 3$).

5. Inhibition of forskolin-stimulated cAMP accumulation in CHO-sst_{2A}, CHO-sst_{2A+5} and AtT-20 cells

To determine whether pre-stimulation with [D-Trp^8]-SRIF-14 or L-779,976 induces functional desensitization, we measured the ability of cells to inhibit forskolin-stimulated cAMP production. Following a 40 min pre-stimulation with either [D-Trp^8]-SRIF-14 or L-779,976 at 37°C and pH 7.4, cells were given a second stimulation with either forskolin alone, forskolin and [D-Trp^8]-SRIF-14, or forskolin and L-779,976 (Fig. 7). Cells receiving no pre-stimulation followed by forskolin alone exhibited an approximate 20-25-fold increase in intracellular cAMP production (data not shown).

[D-Trp^8]-SRIF-14 inhibited forskolin-stimulated cAMP production by 42, 43 and 40% in CHO-sst_{2A}, CHO-sst_{2A+5} and AtT-20 cells, respectively, that received no pre-stimulation (Fig. 7*A, B, C, white bars*). In all cell types, pre-stimulation with [D-Trp^8]-SRIF-14 resulted in a decrease in responsiveness upon a second stimulation with [D-Trp^8]-SRIF-14, resulting in only 7-13% decrease in cAMP levels (Fig 7*A, B, C, grey bars*; $p \leq 0.05$, $p \leq 0.01$; $p \leq 0.001$; $n = 4$). These results suggest that continuous stimulation with [D-Trp^8]-SRIF-14 promotes functional desensitization.

In CHO-sst_{2A} cells, pre-stimulation with L-779,976 also resulted in a decrease in responsiveness to [D-Trp^8]-SRIF-14 and L-779,976 (Fig. 7*A, black bar* and *hatched bar*, respectively; $p \leq 0.01$; $n = 4$). In contrast,

CHO-sst_{2A+5} and AtT-20 cells that were pre-treated with L-779,976 were still responsive upon further stimulation with [D-Trp^8]-SRIF-14 (Fig. 5, *B, C black bars*). These cells inhibited forskolin-stimulated cAMP production by approximately 35%, indicating that they were not desensitized by L-779,976. Taken together, these results suggest that continuous stimulation with L-779,976 does not promote functional desensitization in cells that express both sst_{2A} and sst_5. However, inhibition of cAMP production was markedly reduced in CHO-sst_{2A+5} and AtT-20 cells that received L-779,976 in the second stimulation (Fig. 5, *B, C hatched bars*; $p \leq 0.05$, $p \leq 0.01$; $n = 4$) as compared with those that received [D-Trp^8]-SRIF-14, suggesting that these cells were not as responsive to L-779,976 as they were to [D-Trp^8]-SRIF-14.

6. Trafficking of sst_5 in CHO-sst_5, CHO-sst_{2A+5} and AtT-20 cells

To characterize the agonist-induced internalization and trafficking of HA-tagged sst_5 receptor in CHO-sst_5 and CHO-sst_{2A+5} cells and the endogenously expressed sst_5 in AtT-20 cells, we conducted immunocytochemical analysis. In the absence of agonist, sst_5 immunostaining was distributed throughout the cytoplasm in puncti-like clusters with some perinuclear concentration in CHO-sst_5 (Fig. 8*A* and *B*) CHO-sst_{2A+5} (Fig. 8*I* and *J*) and AtT-20 cells (Fig. 9*A* and *B*). Furthermore, there was limited sst_5 immunostaining at the cell periphery in all cell types. Following 5 min of stimulation with [D-Trp^8]-SRIF-14, there was no apparent change in CHO-sst_5 (Fig. 8*C*) CHO-sst_{2A+5} (Fig. 8*K*) or AtT-20 cells (Fig. 9*C*). However, after 20 and 40 min of simulation, perinuclear accumulation of immunofluorescent puncti resembling endosomes was evident (Fig. 8*E, G, M, O*; Fig. 9*E, G*).

To investigate whether selective stimulation of sst_{2A} affects sst_5 trafficking, we examined sst_5 immunostaining in CHO-sst_5, CHO-sst_{2A+5} and AtT-20 cells following stimulation with L-779,976. A small increase in peripheral sst_5 immunofluorescence was observed in CHO-sst_{2A+5} cells following stimulation with L-779,976 (Fig. 8*N, P*). This increase was not observed in CHO-sst_5 cells (Fig. 8*F, H*), which provides qualitative evidence to suggest that selective stimulation of sst_{2A} may affect the trafficking of sst_5 receptors. However this increase in peripheral sst_5 distribution was not as apparent in AtT-20 cells (Fig. 9*F, H*).

7. Double immunolabeling of sst_{2A} and sst_5

To determine whether c-Myc-sst_{2A} and HA-sst_5 receptors co-localize following agonist stimulation, we performed double immunolabeling on permeablized CHO-sst_{2A+5} cells. Confocal images suggest that in the absence of ligand (Fig. 10*A-C*), these two SRIF receptor subtypes are distributed in different cellular regions. While sst_{2A} immunostaining was localized at the cell periphery, sst_5 was distributed in the cytoplasm with little peripheral sst_5 staining. Following 40 min of stimulation with [D-Trp^8]-SRIF-14 (Fig. 10*D-F*), there was intracellular accumulation of sst_{2A} and sst_5 receptors in punctiform compartments resembling endosomes. However, the absence of overlap between sst_{2A} and sst_5 immunopositive particles indicates that these receptor subtypes do not co-localize in the same compartments. Following stimulation with L-779,976 (Fig. 10*G-I*), sst_{2A} was located at the cell periphery, and while there was a small increase in peripheral sst_5, the limited overlap between sst_{2A} and sst_5 immunostaining suggests that they were not co-localized. Taken together, these results suggest that there is no physical association between sst_{2A} and sst_5 receptors neither before nor after agonist stimulation.

Table 1

Table 1. Agonist binding affinities

Cell Type	IC_{50} (*nM*) [D-Trp^8]-SRIF-14	L-779,976
CHO-sst_{2A}	0.54 (± 0.02)	32 (± 0.38)
CHO-sst_5	61 (± 0.98)	ND*
CHO-sst_{2A+5}	0.19 (± 0.03)	50 (± 0.52)
AtT-20	1.7 (± 0.16)	5.7 (± 0.30)

**up to 10^{-7} M*

IC_{50}s for [D-Trp^8]-SRIF-14 and L-779,976 in CHO-K1 cells stably transfected with mouse c-Myc-sst_{2A} (CHO-sst_{2A}), HA-sst_5 (CHO-sst_5), both receptor constructs (CHO-sst_{2A+5}), and non-transfected AtT-20 cells. Displacement binding experiments were done on whole live cells at 37°C and pH 7.4 using 0.3 nM ^{125}I-Tyr^0-[D-Trp^8]-SRIF-14 diluted with increasing concentrations (10^{-12} to 10^{-6} M) of unlabeled [D-Trp^8]-SRIF-14 or L-779,976. Each value represents the mean ± SEM of at least two independent experiments done in triplicate for each ligand.

Table 2

Table 2. A summary of cell surface ^{125}I-Tyr^0[D-Trp^8]-SRIF-14 binding and sst_{2A} immunofluorescence quantification data.

Cell Type	Remaining Cell surface Binding Sites		Remaining Cell Surface Sst_{2A} Immunoreactivity	
	[D-Trp^8]-SRIF-14	L-779,976	[D-Trp^8]-SRIF-14	L-779,976
CHO-sst_{2A}	20%	25%	25%	30%
CHO-sst_{2A+5}	25%	**60%**	20%	**40%**
AtT-20	40%	**75%**	35%	**55%**

Remaining cell surface receptor density measurements following 40 min of agonist stimulation from radioactive ligand binding and sst_{2A} immunofluorescence quantification experiments. See *Materials and Methods*, pages 40-45 for statistical methodology.

Figure 1

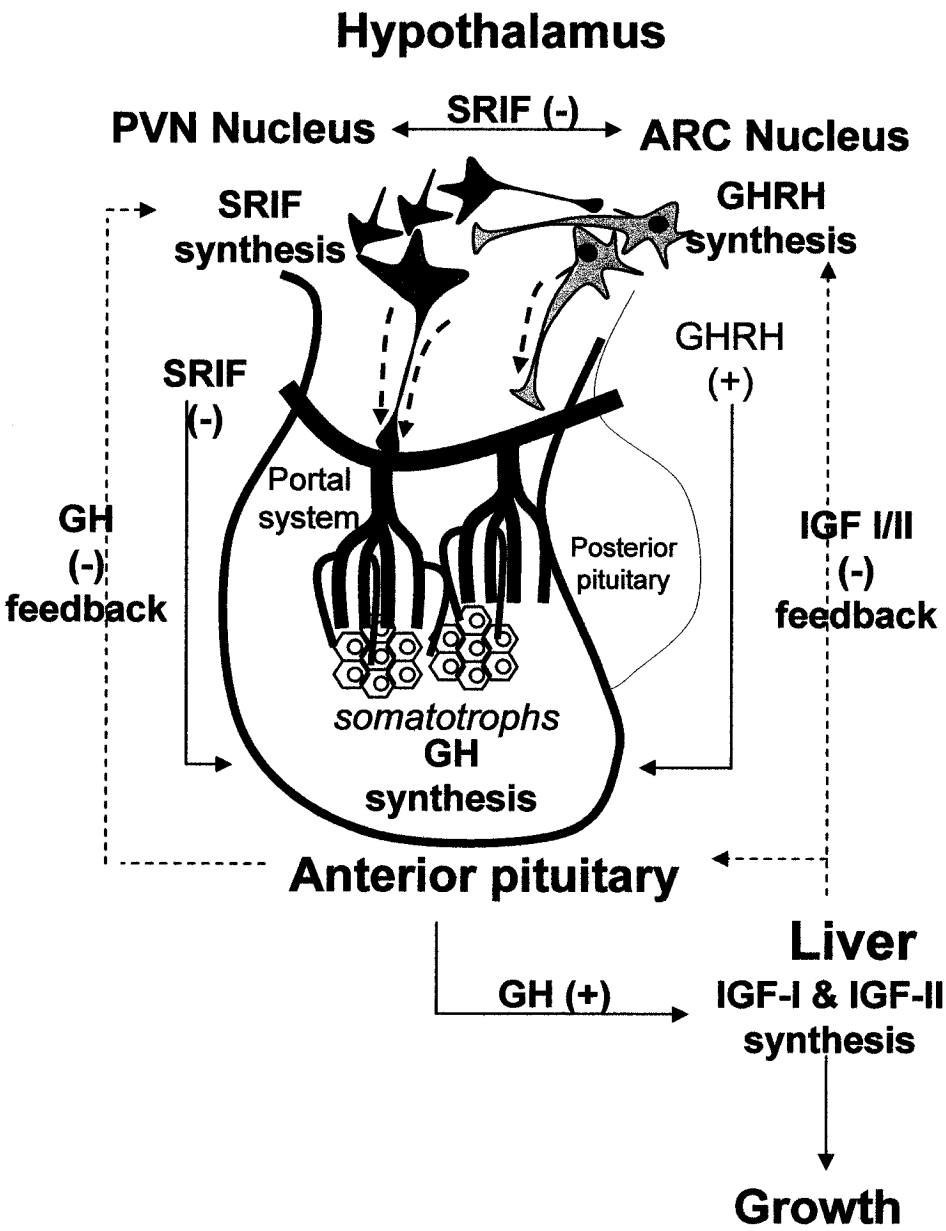

Hypothalamus
PVN Nucleus
SRIF (-)
ARC Nucleus
SRIF synthesis
GHRH synthesis
SRIF (-)
GHRH (+)
Portal system
Posterior pituitary
GH (-) feedback
IGF I/II (-) feedback
somatotrophs
GH synthesis
Anterior pituitary
Liver
GH (+)
IGF-I & IGF-II synthesis
Growth

Figure 2

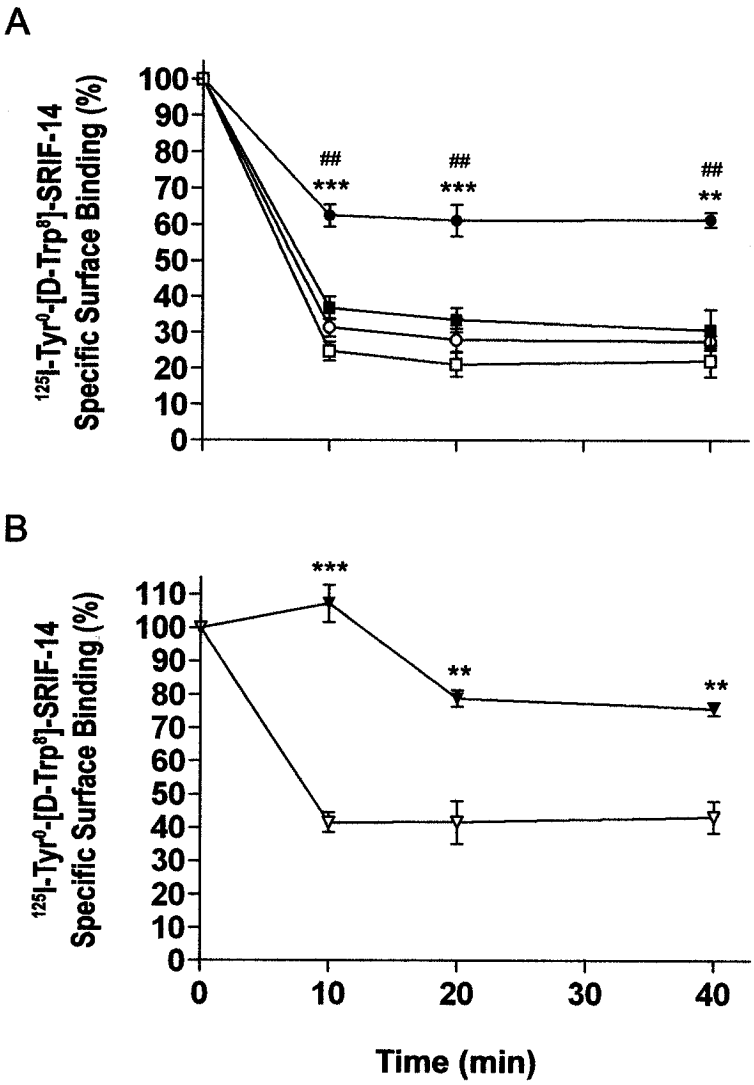

A
B
^{125}I-Tyr^0-[D-Trp^8]-SRIF-14
Specific Surface Binding (%)
100
90
80
70
60
50
40
30
20
10
0
##
***
**
110
0
10
20
30
40
Time (min)

Figure 3

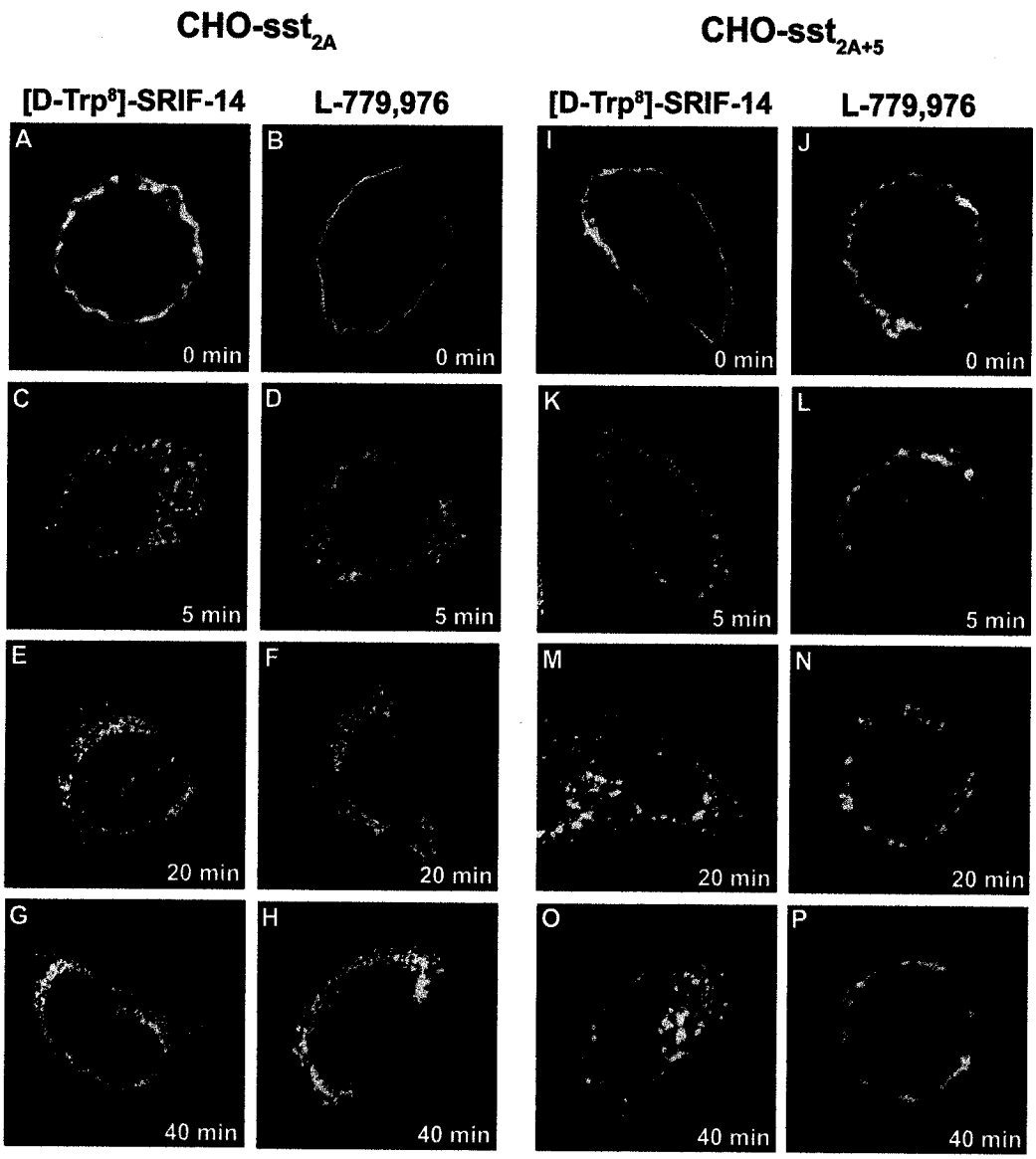
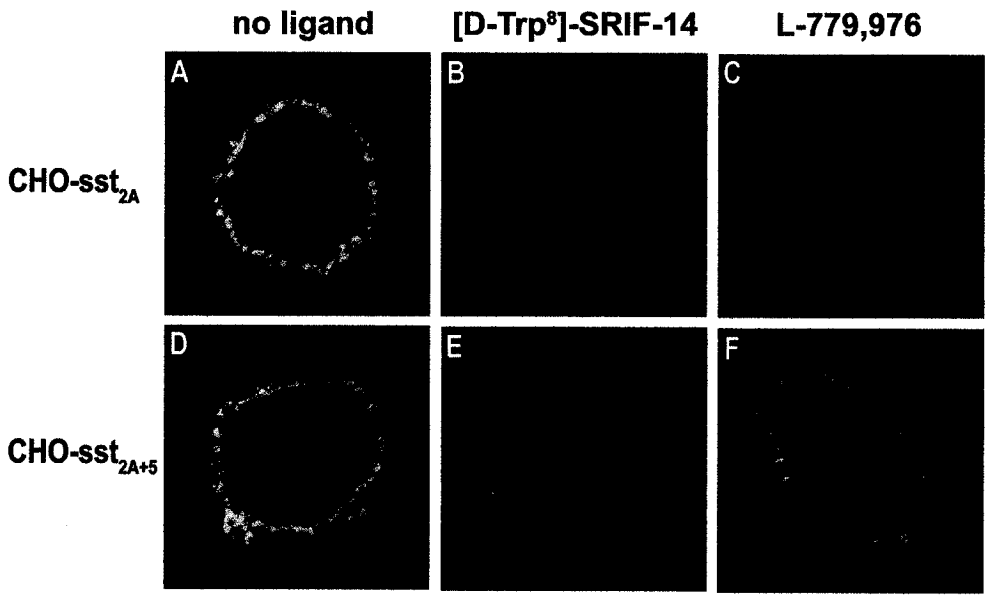

CHO-sst2A
CHO-sst2A+5
[D-Trp8]-SRIF-14
L-779,976
[D-Trp8]-SRIF-14
L-779,976
A
B
I
J
0 min
0 min
0 min
0 min
C
D
K
L
5 min
5 min
5 min
5 min
E
F
M
N
20 min
20 min
20 min
20 min
G
H
O
P
40 min
40 min
40 min
40 min

Figure 4


no ligand
[D-Trp8]-SRIF-14
L-779,976
A
B
C
D
E
F
CHO-sst2A
CHO-sst2A+5

G

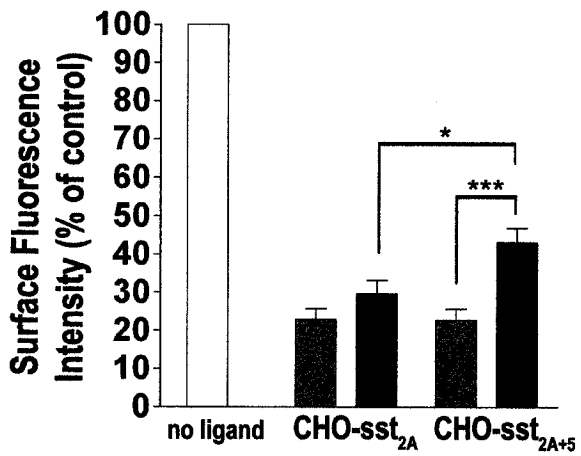

Surface Fluorescence Intensity (% of control)
100
90
80
70
60
50
40
30
20
10
0
*
***
no ligand
CHO-sst2A
CHO-sst2A+5

Figure 5

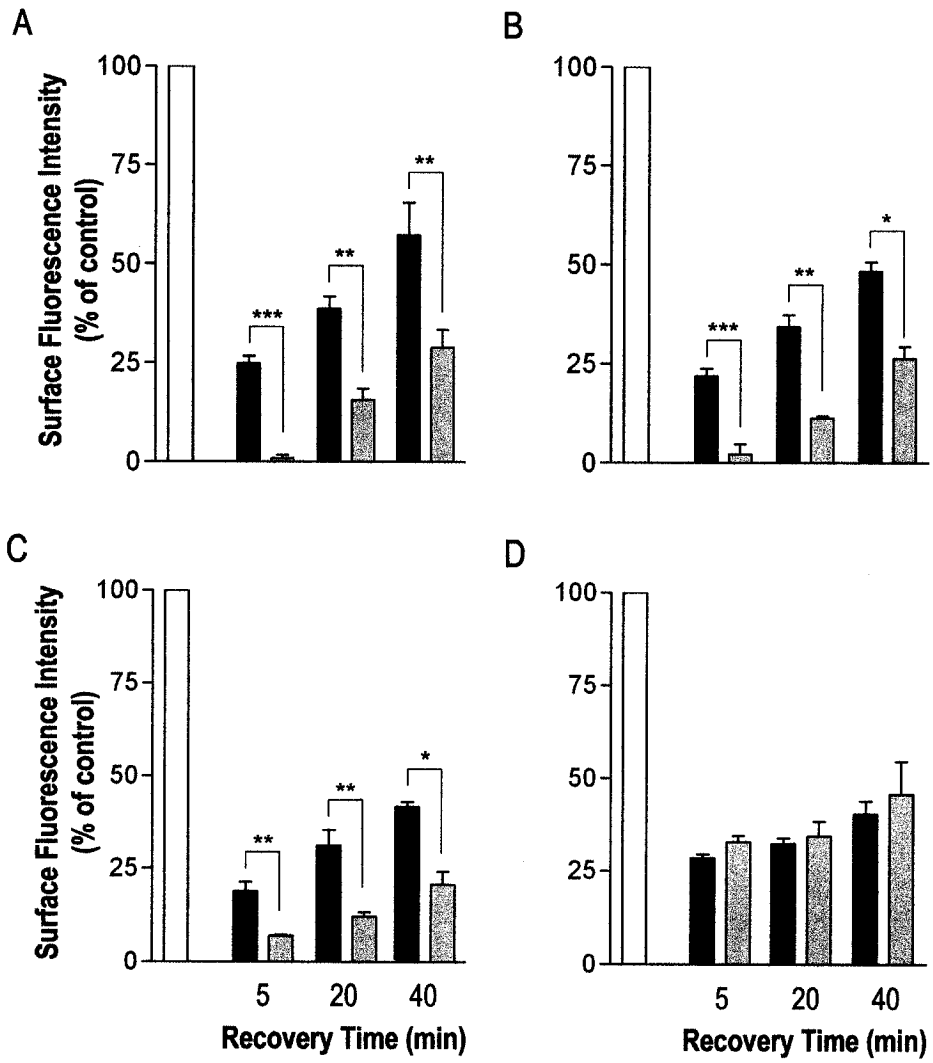
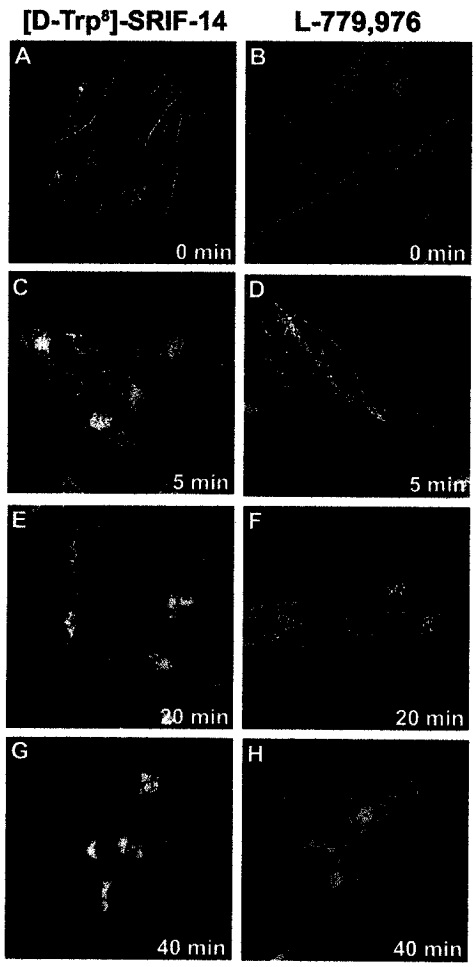

A
B
C
D
Surface Fluorescence Intensity (% of control)
100
75
50
25
0
***
**
*
5
20
40
Recovery Time (min)

Figure 6


[D-Trp⁸]-SRIF-14
L-779,976
A
0 min
B
0 min
C
5 min
D
5 min
E
20 min
F
20 min
G
40 min
H
40 min

I

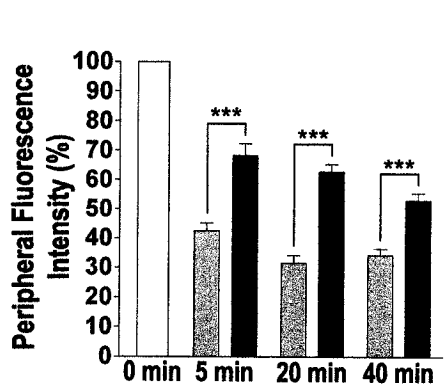

Peripheral Fluorescence Intensity (%)
100
90
80
70
60
50
40
30
20
10
0
***
***
***
0 min
5 min
20 min
40 min

Figure 7

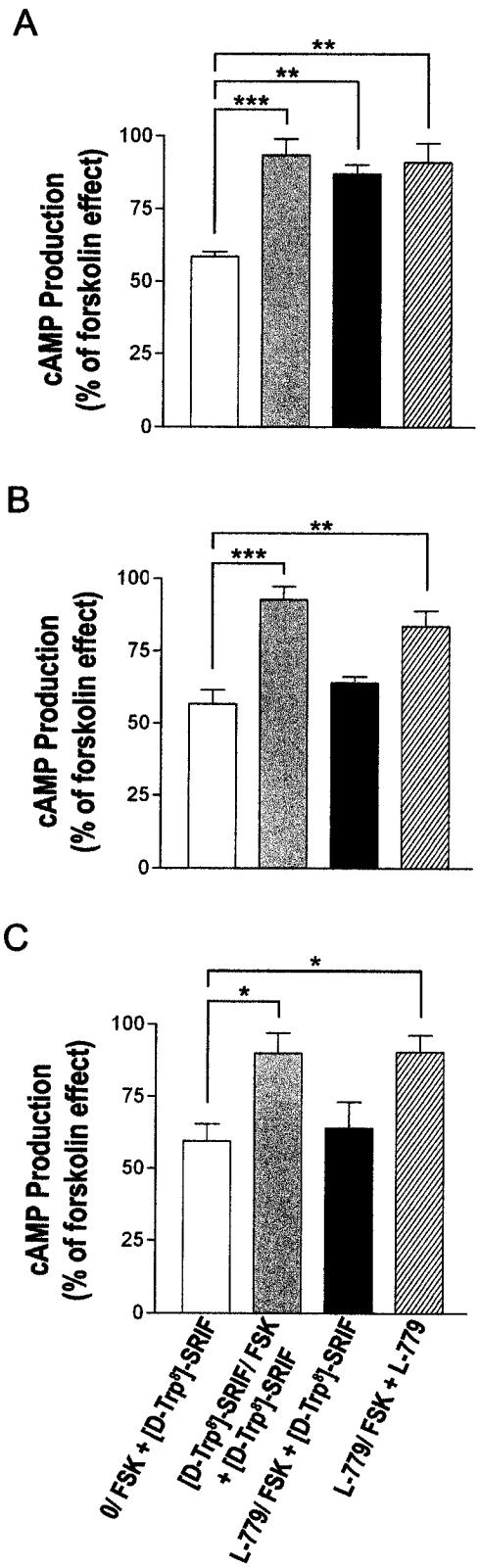

A
B
C
cAMP Production
(% of forskolin effect)
100
75
50
25
0
**
**
***
***
**
*
*
0/ FSK + [D-Trp8]-SRIF
[D-Trp8]-SRIF/ FSK
+ [D-Trp8]-SRIF
L-779/ FSK + [D-Trp8]-SRIF
L-779/ FSK + L-779

Figure 8

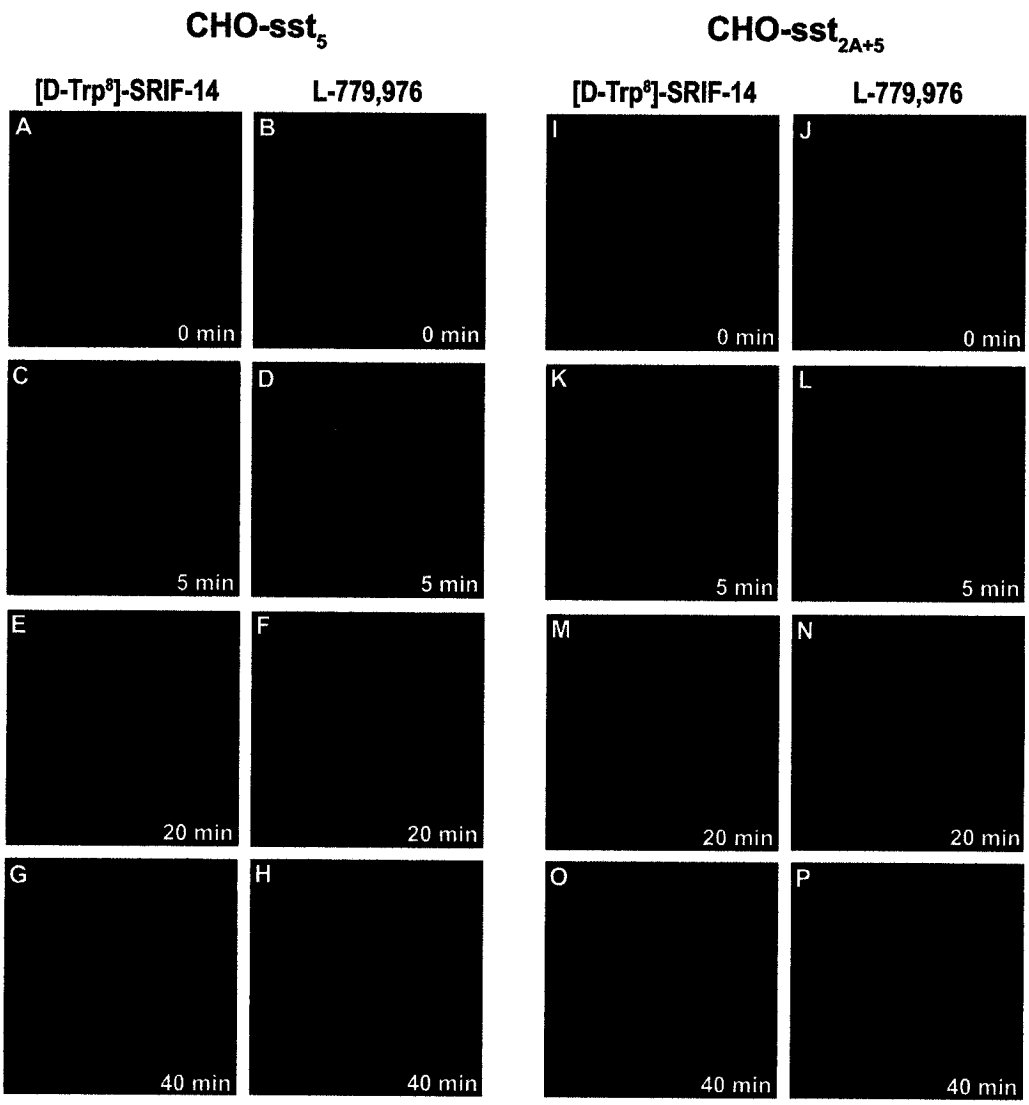

CHO-sst5
CHO-sst2A+5
[D-Trp8]-SRIF-14
L-779,976
[D-Trp8]-SRIF-14
L-779,976
A
0 min
B
0 min
I
0 min
J
0 min
C
5 min
D
5 min
K
5 min
L
5 min
E
20 min
F
20 min
M
20 min
N
20 min
G
40 min
H
40 min
O
40 min
P
40 min

Figure 9

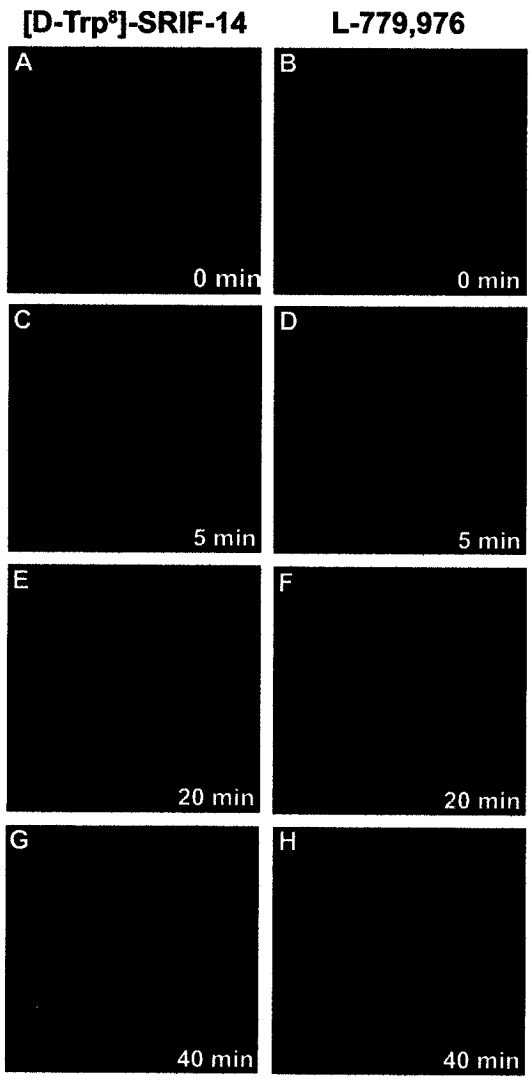

[D-Trp⁸]-SRIF-14
L-779,976
A
0 min
B
0 min
C
5 min
D
5 min
E
20 min
F
20 min
G
40 min
H
40 min

Figure 10

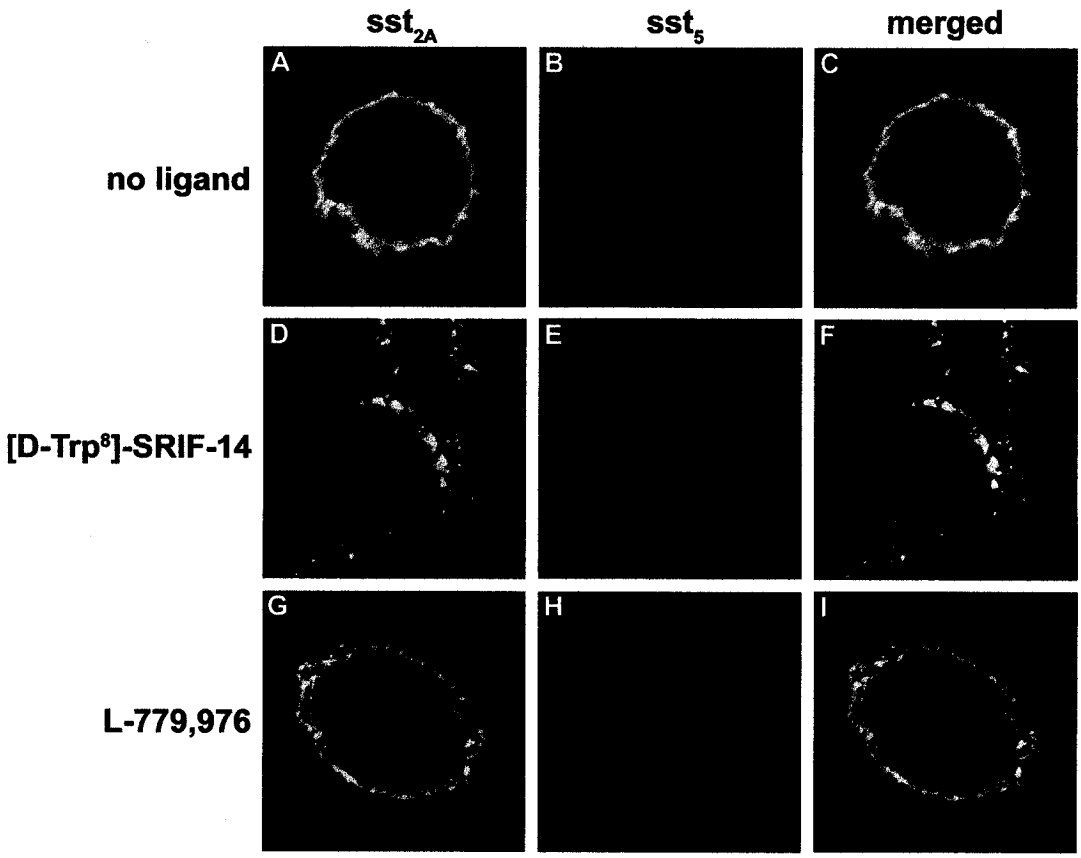

sst2A
sst5
merged
A
B
C
no ligand
D
E
F
[D-Trp8]-SRIF-14
G
H
I
L-779,976

Figure 11

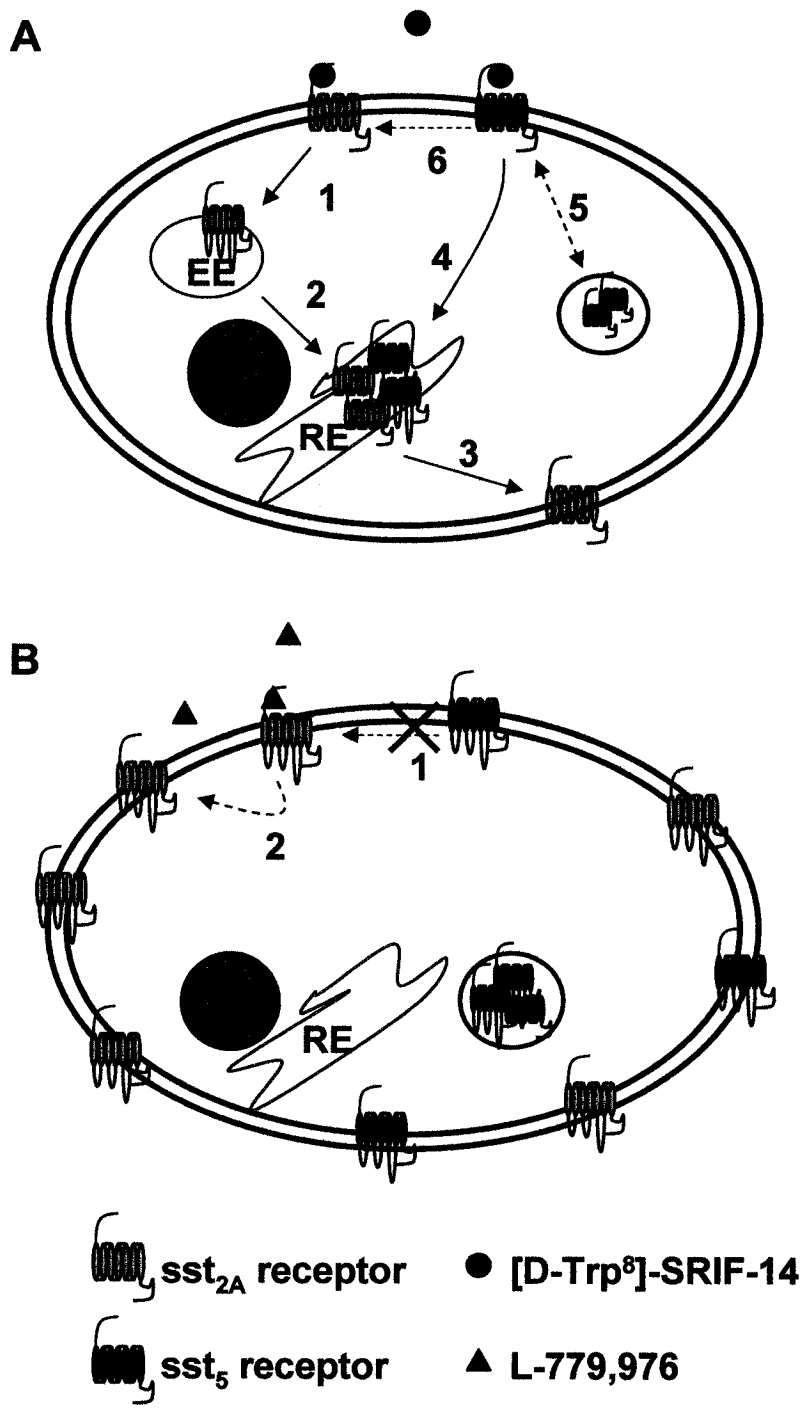

A
6
1
5
EE
4
2
RE
3
B
1
2
RE
sst2A receptor
[D-Trp8]-SRIF-14
sst5 receptor
L-779,976

Figure Legends

Figure 1. **A schematic diagram of the hypothalamo-pituitary axis.**

Figure. 2. **Cell surface ^{125}I-Tyr^0-[D-Trp^8]-SRIF-14 binding to CHO-sst$_{2A}$, CHO-sst$_{2A+5}$ and AtT-20 cells.** Following 0-40 min of stimulation with 100 nM of [D-Trp^8]-SRIF-14 or L-779,976 at 37°C and pH 7.4, a hypertonic acid wash was performed to strip off surface-bound ligand, and cells were incubated with 0.3 nM ^{125}I-Tyr^0-[D-Trp^8]-SRIF-14 at 37°C in the presence of PAO (to inhibit receptor internalization). *A*, Agonist-induced reduction of binding sites in CHO-sst$_{2A}$ and CHO-sst$_{2A+5}$ cells. Stimulation with [D-Trp^8]-SRIF-14 induces a decrease in surface binding sites in CHO-sst$_{2A}$ (□) and CHO-sst$_{2A+5}$ cells (○). While L-779,976 induces a decrease in surface binding sites in both cell types, significantly more binding sites remain in CHO-sst$_{2A+5}$ (●) as compared with [D-Trp^8]-SRIF-14 stimulation in the same cell type (○, ##, $p \leq 0.01$) and with L-779,976 stimulation in CHO-sst$_{2A}$ (■; **, $p \leq 0.01$; ***, $p \leq 0.001$). *B*, Agonist-induced reduction of binding sites in AtT-20 cells. While [D-Trp^8]-SRIF-14 stimulation induces a decrease in surface binding sites (▽), this effect is considerably less during stimulation with L-779,976 (▼; **, $p \leq 0.01$; ***, $p \leq 0.001$). Data represent the mean ± SEM of five (*A*) or four (*B*) independent experiments done in triplicate, and are expressed as a percentage of specific surface binding in non-stimulated cells.

Figure 3. **Agonist-induced internalization and trafficking of c-Myc-sst_{2A} in CHO-sst_{2A} and CHO-sst_{2A+5} cells.** Following 0-40 min of stimulation with 100 nM of [D-Trp^8]-SRIF-14 or L-779,976 at 37°C and pH 7.4, cells were fixed, permeablized and incubated with a primary antibody directed against the c-Myc epitope tag. *A-H (CHO-sst_{2A})*, in the absence of ligand (0 min), sst_{2A} is localized at the cell periphery (*A*, *B*). Stimulation with [D-Trp^8]-SRIF-14 or L-779,976 (*C-H*) induces rapid internalization and intracellular accumulation of sst_{2A} in the perinuclear region. *I-P (CHO-sst_{2A+5})*, In the absence of ligand, sst_{2A} is localized at the cell periphery (*I*, *J*). While stimulation with [D-Trp^8]-SRIF-14 results in rapid sst_{2A} internalization and perinuclear accumulation (*K, M, O*), sst_{2A} remains in the cell periphery following L-779,976 stimulation (*L, N, P*), and there is only limited perinuclear accumulation.

Figure 4. **Quantification of cell surface c-Myc-sst_{2A} immunofluorescence following agonist stimulation in CHO-sst_{2A}, CHO-sst_{2A+5}.** Following 40 min of stimulation with 100 nM of [D-Trp^8]-SRIF-14 or L-779,976 at 37°C and pH 7.4, CHO-sst_{2A} and CHO-sst_{2A+5} cells were incubated with an anti-c-Myc antibody for 90 min at 4°C to label cell surface sst_{2A} receptors, and immunofluorescence was analyzed either using a fluorescence plate reader or a confocal microscope. *A-F*, disappearance of cell surface c-Myc-sst_{2A} immunofluorescence in CHO-

sst_{2A} and CHO-sst_{2A+5}. In the absence of ligand, sst_{2A} immunofluorescence is observed at the cell surface in both CHO-sst_{2A} (*A*) and CHO-sst_{2A+5} cells (*D*). Following stimulation with [D-Trp^8]-SRIF-14 little cell surface sst_{2A} immunofluorescence remains in both cell lines (*B*, CHO-sst_{2A} cells; *E*, CHO-sst_{2A+5} cells). While L-779,976 induces a reduction of cell surface sst_{2A} in both cell types, this effect is much reduced in CHO-sst_{2A+5} (*F*) as compared with CHO-sst_{2A} cells (*C*) or with [D-Trp^8]-SRIF-14 stimulation in either cell type. *G*, quantification of cell surface sst_{2A} immunofluorescence. *White bar*, non-stimulated control cells; *grey bars*, [D-Trp^8]-SRIF-14 stimulation; *black bars*, L-779,976 stimulation; each bar represents the mean ± SEM of three independent experiments done in quadruplicate, and is expressed as a percentage of the cell surface fluorescence in non-stimulated cells (*, $p \leq 0.05$; ***, $p \leq 0.001$).

Figure 5. **Recovery of cell surface c-Myc-sst_{2A} immunofluorescence in CHO-sst_{2A} and CHO-sst_{2A+5} cells.** Following 40 min of stimulation with 100 nM of [D-Trp^8]-SRIF-14 or L-779,976 at 37°C and pH 7.4, an acid wash was performed and cells were incubated in fresh Earle's buffer over a range of recovery periods (0-40 min). Cells were then incubated with an anti-c-Myc antibody for 90 min at 4°C to reveal cell surface sst_{2A} receptors, and immunofluorescence was quantified on a fluorescence plate reader. *A* and *B*, in CHO-sst_{2A} cells, re-appearance of cell surface sst_{2A}

immunofluorescence increases with time (*black bars*) following stimulation with [D-Trp^8]-SRIF-14 (*A*) and L-779,976 (*B*). In the presence of monensin (*grey bars*), re-appearance of cell surface sst_{2A} immunofluorescence is reduced. *C* and *D*, in CHO-sst_{2A+5} cells, re-appearance of cell surface sst_{2A} immunofluorescence increases with time following stimulation with [D-Trp^8]-SRIF-14 (*C, black bars*) and is reduced by monensin (*C, grey bars*). However, following stimulation with L-779,976, there is little increase in cell surface sst_{2A} immunofluorescence (*D, black bars*) and monensin has no effect (*grey bars*). Each bar represents the mean ± SEM of three independent experiments done in quadruplicate, and is expressed as a percentage of the cell surface fluorescence in non-stimulated cells.

Figure 6. **Agonist-induced internalization and trafficking of sst_{2A} in AtT-20 cells.** Following 0-40 min of stimulation with 100 nM of [D-Trp^8]-SRIF-14 or L-779,976 at 37°C and pH 7.4, cells were fixed, permeablized, and incubated with a primary antibody directed against the C-terminus of the sst_{2A} receptor. In the absence of ligand (0 min), sst_{2A} is localized primarily at the cell periphery (*A*, *B*). Stimulation with [D-Trp^8]-SRIF-14 (*C*, *E*, *G*) results in a loss of peripheral sst_{2A} and accumulation of immunoreactivity in the perinuclear region. In contrast, upon stimulation with L-779,976 (*D*, *F*, *H*), sst_{2A} immunofluorescence remains largely peripheral and shows limited perinuclear accumulation. *I*, quantification of peripheral sst_{2A} immunofluorescence. *White bar*, non-stimulated control

cells; *grey bars*, [D-Trp^8]-SRIF-14 stimulation; *black bars*, L-779,976 stimulation; each bar represents the mean ± SEM of three independent experiments done in quadruplicate, and is expressed as a percentage of the peripheral fluorescence in non-stimulated cells (***, $p \leq 0.001$).

Figure 7. **Inhibition of forskolin-stimulated cAMP production in CHO-sst_{2A}, CHO-sst_{2A+5} and AtT-20 cells.** Cells were pre-stimulated for 40 min with 100 nM of either [D-Trp^8]-SRIF-14 or L-779,976 at 37°C and pH 7.4, and the inhibition of forskolin-stimulated cAMP production by a second stimulation with [D-Trp^8]-SRIF-14 or L-779,976 was determined. Following no pre-stimulation (*white bars*) [D-Trp^8]-SRIF-14 inhibits forskolin-stimulated cAMP production in all cell types (*A*, CHO-sst_{2A} cells; *B*, CHO-sst_{2A+5} cells; *C*, AtT-20 cells). Pre-stimulation of CHO-sst_{2A} cells (*A*) with [D-Trp^8]-SRIF-14 (*grey bar*) or L-779,976 (*black bar*) desensitizes cells to further stimulation with [D-Trp^8]-SRIF-14 (*grey bar and black bar*) or L-779,976 (*hatched bar*). While pre-stimulation of CHO-sst_{2A+5} (*B*) and AtT-20 cells (*C*) with [D-Trp^8]-SRIF-14 desensitizes them to further [D-Trp^8]-SRIF-14 stimulation (*grey bars*), pre-stimulation with L-779,976 does not (*black bars*). This effect is greatly reduced in cells that receive a second stimulation with L-779,976 (*hatched bars*). Each bar represents the mean ± SEM of four independent experiments done in quadruplicate, and is expressed as a percentage of the effect of forskolin alone in the second stimulation (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

Figure 8. **Agonist-induced trafficking of HA-sst_5 in CHO-sst_5 and CHO-sst_{2A+5} cells.** Following 0-40 min of stimulation with 100 nM of [D-Trp^8]-SRIF-14 or L-779,976 at 37°C and pH 7.4, cells were fixed, permeablized and incubated with a primary antibody directed against the HA epitope tag. *A-H (CHO-sst_5)*, in the absence of ligand (0 min), sst_5 is distributed throughout the cytoplasm, and is limited at the cell periphery (*A*, *B*). While stimulation with [D-Trp^8]-SRIF-14 (*C, E, G*) induces intracellular accumulation of sst_5 receptors in the perinuclear region, stimulation with L-779,976 (*D, F, H*) does not. *I-P (CHO-sst_{2A+5})*, In the absence of ligand, sst_5 is localized throughout the cytoplasm (*I*, *J*). Stimulation with [D-Trp^8]-SRIF-14 (*K, M, O*) results in perinuclear accumulation of sst_5 receptors, however L-779,976 stimulation (*L, N, P*) results in a small increase in peripheral sst_5 immunofluorescence.

Figure 9. **Agonist-induced trafficking of sst_5 in AtT-20 cells.** Following 0-40 min of stimulation with 100 nM of [D-Trp^8]-SRIF-14 or L-779,976 at 37°C and pH 7.4, cells were fixed, permeablized and incubated with a primary antibody directed against the C-terminus of the sst_5 receptor. In the absence of ligand (0 min), sst_5 is distributed throughout the cytoplasm and at the cell periphery (*A*, *B*). Stimulation with [D-Trp^8]-SRIF-14 (*C, E, G*) induces intracellular accumulation of sst_5 receptors in the perinuclear region, whereas L-779,976 (*D, F, H*) does not.

Figure 10. **Double immunolabeling of c-Myc-sst_{2A} and HA-sst_5 in CHO-sst_{2A+5} cells.** Following 40 min of stimulation with 100 nM of [D-Trp^8]-SRIF-14 or L-779,976 at 37°C and pH 7.4, cells were fixed, permeablized and incubated with primary antibodies directed against the c-Myc and HA epitope tags. *A-C*, in the absence of ligand sst_{2A} is located at the periphery of the cell and sst_5 is distributed throughout the cytoplasm; there is no co-localization. *D-F*, following stimulation with [D-Trp^8]-SRIF-14, sst_{2A} and sst_5 immunopositive puncti are distributed throughout the cytoplasm and accumulate in the perinuclear region. However, each receptor subtype is localized in distinct vesicular compartments. *G-I*, following stimulation with L-779,976, sst_{2A} is localized at the cell periphery while sst_5-immunopositive puncti are largely distributed throughout the cytoplasm. There is no overlap between sst_{2A} and sst_5 immunostaining.

Figure 11. **A model for the regulation of sst_{2A} and sst_5 in cells that express both receptor subtypes.** A schematic model depicting the cellular regulation of sst_{2A} and sst_5 following stimulation with [D-Trp^8]-SRIF-14 (*A*) or L-779,976 (*B*). This model is based on experimental observations and hypothetical mechanisms. EE: early endosomes; N: nucleus; RE: pericentriolar recycling endosome.

Discussion

In the present study, we have demonstrated that selective stimulation of sst_{2A} receptors with L-779,976 in cells that express both sst_{2A} and sst_5 results in a completely different pattern of sst_{2A} internalization, intracellular trafficking and signaling as compared with cells that express sst_{2A} alone. Furthermore, this effect was not observed following stimulation with [D-Trp^8]-SRIF-14, suggesting that selective stimulation of sst_{2A} induces distinct regulatory events. Taken together, our findings indicate that the presence of sst_5 receptors influences the cellular regulation of sst_{2A} receptors and their responsiveness to SRIF agonists.

L-779,976 is a synthetic, non-peptide agonist that has been reported to bind to sst_2 receptors with the same affinity as SRIF-14, and to be highly selective for sst_2 over other SRIF receptor subtypes (Rohrer *et al.*, 1998). Nevertheless, we wanted to verify the binding affinity of this compound in our cell models and under the relevant experimental conditions. We showed that L-779,976 does not bind to sst_5 over a wide range of concentrations up to 100 nM. However, our results suggest that L-779,976 binds to sst_{2A} receptors with considerably lower affinity than does [D-Trp^8]-SRIF-14. The discrepancy between the binding affinity to sst_{2A} that we have determined for L-779,976 in whole live cells and those in membrane preparations of transfected cells (e.g. Rohrer *et al.*, 1998) are likely due to differing experimental manipulations. Therefore, our

findings demonstrate that the binding affinities of synthetic ligands can vary substantially between different experimental conditions. Nevertheless, we confirmed that L-779,976 binds readily to sst_{2A} receptors and that this compound is selective for sst_{2A} over sst_5.

As is the case with many other GPCRs, agonist-induced endocytosis of cell surface sst_{2A} receptors is a clathrin-mediated process that results in an overall reduction in surface receptor density (Koenig *et al.*, 1998; Sarret et al., 1999 Hipken *et al.*, 2000; Stroh *et al.*, 2000a; Brasselet *et al.*, 2002), and intracellular accumulation of sst_{2A} in endosomal compartments (Nouel et al., 1997; Boudin *et al.*, 2000; Stroh *et al.*, 2000a; Csaba *et al.*, 2001; Brasselet *et al.*, 2002; Sarret *et al.*, 2004; Tulipano *et al.*, 2004). In the presence of agonist, the trafficking of SRIF receptors between the cell surface and intracellular compartments reaches a steady state (Koenig and Edwardson, 1997; Koenig, 2004). The remaining cell surface receptor density following agonist stimulation can be quantified using high affinity radioactively labeled ligands or immunocytochemical techniques under conditions that inhibit receptor endocytosis (Koenig, 2004). We used both of these approaches to investigate whether the presence of sst_5 affects the agonist-induced regulation of sst_{2A}.

In agreement with our previous reports (Nouel et al., 1997; Stroh *et al.*, 2000a; Sarret *et al.*, 1999; Sarret *et al.*, 2004), our present findings demonstrate that stimulation with [D-Trp^8]-SRIF-14 induces a drastic

reduction in cell surface sst_{2A} density in cells expressing sst_{2A} alone and in cells co-expressing sst_5. Internalization of sst_{2A} was followed by intracellular accumulation of the receptor in a juxtanuclear compartment previously identified as the TGN-pericentriolar recycling endosome (Sarret *et al.*, 2004). In cells expressing sst_{2A} alone, stimulation with L-779,976 induced the same pattern of sst_{2A} internalization and perinuclear accumulation as did [D-Trp^8]-SRIF-14. In the absence of agonist, sst_{2A} was recycled back to the cell surface and this process was pH-dependent, suggesting the involvement of early endosomes. However, the re-appearance of cell surface sst_{2A} receptors was not completely blocked by monensin, therefore it is likely that spare and/or newly synthesized sst_{2A} receptors were also targeted to the cell surface.

In a recent study, Liu and colleagues (2005) reported that SRIF-14 was more efficacious than L-779,976 at inducing internalization of sst_{2A} receptors, and suggested that this may be due to distinct receptor conformational changes induced by this agonist. Indeed, non-peptide agonists targeting other GPCRs such as the opioid receptors have been shown to be less proficient than peptide agonists at inducing endocytosis (von Zastrow et al., 2004). For example, while the peptide agonist DADLE induces rapid internalization of cell surface δ and μ opioid receptors, morphine, an alkaloid agonist, does not (Keith *et al.*, 1996). In the present study, while radioactive ligand binding revealed that stimulation with [D-Trp^8]-SRIF-14 induced slightly more cell surface sst_{2A} receptor reduction

than did L-779,976 (less than 10% after 40 min), this difference was likely due to the approximate 100-fold greater affinity of [D-Trp^8]-SRIF-14 over L-779,976 for sst_{2A} in CHO-sst_{2A} cells. Therefore, taken together our results suggest that L-779,976 is as proficient as [D-Trp^8]-SRIF-14 at inducing sst_{2A} internalization in cells expressing sst_{2A} alone.

In stark contrast to our observations in CHO-sst_{2A} cells, stimulation of CHO-sst_{2A+5} with L-779,976 resulted in a relative preservation of cell surface binding sites and sst_{2A} immunoreactivity. Since we demonstrated that L-779,976 is as efficacious as [D-Trp^8]-SRIF-14 at inducing sst_{2A} internalization in CHO-sst_{2A} cells, it is unlikely that our observations in CHO-sst_{2A+5} cells were due to differing pharmacological properties of the two agonists. Rather, our results indicate that the presence of sst_5 affects the density of sst_{2A} receptors at the cell surface. Furthermore, since dual stimulation of sst_{2A} and sst_5 with [D-Trp^8]-SRIF-14 induced a different pattern of sst_{2A} internalization and trafficking than did selective stimulation of sst_{2A} with L-779,976, our findings also suggest that the activation of sst_5 affects the cellular regulation of sst_{2A} receptors.

In AtT-20 cells, more cell surface binding sites remained following continuous stimulation with L-779,976 than with [D-Trp^8]-SRIF-14. In fact, there was no net loss of cell surface binding sites within the first 10 min of L-779,976 stimulation, suggesting that cell surface receptor density is maintained following stimulation with this agonist. Moreover, similar to what was observed in CHO-sst_{2A+5} cells, L-779,976 stimulation induced

less reduction of peripheral sst_{2A} immunoreactivity than did stimulation with [D-Trp^8]-SRIF-14. While there is evidence suggesting that sst_1 and sst_4 receptors may also be expressed in AtT-20 cells (Patel *et al.*, 1994; Sarret *et al.*, 1999), several recent studies have demonstrated that this cell line expresses specific binding sites for only sst_2 and sst_5 receptors (Strowski *et al.*, 2002; Cervia *et al.*, 2000a; 2003b). Therefore, our findings in AtT-20 cells suggest that the endogenous expression of sst_5 affects the internalization and trafficking of sst_{2A} receptors.

To quantify cell surface receptor density following stimulation with [D-Trp^8]-SRIF-14 or L-779,976, we used ^{125}I-Tyr^0[D-Trp^8]-SRIF-14, a ligand that binds with high affinity to both sst_{2A} and sst_5 receptors, and thus cannot distinguish between sst_{2A} and sst_5 binding sites. Our immunocytochemical manipulations, in contrast, allowed us to characterize sst_{2A} and sst_5 individually. A comparison between the data we obtained using these two techniques reveals an interesting discrepancy (see Table 2). In both CHO-sst_{2A+5} and AtT-20 cells, there was approximately 20% more remaining cell surface binding sites than there were immunoreactive sst_{2A} receptors following 40 min of stimulation with L-779,976. This difference was not observed following stimulation with [D-Trp^8]-SRIF-14, an agonist that induces internalization of both sst_{2A} and sst_5 receptors, nor was it observed in CHO-sst_{2A} cells. Therefore, it is possible that the discrepancy between our radioactive ligand binding and

immunofluorescence data was due to the presence of sst_5 receptors at the cell surface.

To investigate whether the differential pattern of sst_{2A} internalization and trafficking observed in CHO-sst_{2A} versus CHO-sst_{2A+5} and AtT-20 cells was reflected on SRIF signaling, we performed desensitization experiments. Stimulation with SRIF analogs results in a loss of the ability to inhibit cAMP production, i.e. cellular desensitization (Reisine and Takahashi, 1984; Mahy *et al.*, 1988). Previously, Brasselet *et al.* (2002) showed that pre-incubation with SRIF-14 desensitized CHO-sst_{2A} cells to further agonist exposure. In the present study, we verified that SRIF-14 stimulation leads to cellular desensitization in the three cell lines examined. Furthermore, we demonstrated that L-779,976 inhibited cAMP production as effectively as [D-Trp^8]-SRIF-14 in CHO-sst_{2A} cells. These results concur with previous reports that have demonstrated that L-779,976 is a potent inhibitor of cAMP production (e.g. Rohrer *et al.*, 1998; Strowski *et al.*, 2002; Liu *et al.*, 2005). However, we are the first to demonstrate that stimulation with L-779,976 leads to cellular desensitization in CHO-sst_{2A} cells.

While stimulation with [D-Trp^8]-SRIF-14 desensitized CHO-sst_{2A+5} and AtT-20 cells, stimulation with L-779,976 did not. Considering that there were more remaining cell surface receptors following L-779,976 stimulation as compared with [D-Trp^8]-SRIF-14 stimulation, it was not surprising that these cells retained the ability to inhibit cAMP production.

Moreover, since L-779,976 potently desensitized CHO-sst_{2A} cells, it is unlikely that the effect we observed in CHO-sst_{2A+5} and AtT-20 cells was due to the pharmacological properties of L-779,976. Rather, we propose that by promoting the maintenance of cell surface receptor density, the co-expression of sst_5 prolongs the responsiveness of these cells to SRIF agonists.

While stimulation of sst_{2A} results in a loss of cell surface receptor density, the agonist-induced internalization and trafficking of sst_5 receptors is very different. In AtT-20 cells, cell surface sst_5 immunodensity is maintained in the presence of agonist (Sarret *et al.*, 1999; Ben-Shlomo *et al.*, 2005), and similar results have been obtained in sst_5-transfected HEK-293 (Roth *et al.*, 1997; Tulipano *et al.*, 2004) and COS-7 cells (Stroh *et al.*, 2000b). Our previous findings suggest that cell surface sst_5 receptors internalize, recycle quickly back to the cell surface, and that the maintenance of cell surface sst_5 is supported by the mobilization of pre-synthesized receptors (Stroh *et al.*, 2000b). In the present study, CHO-sst_{2A+5} and AtT-20 cells that were pre-stimulated with L-779,976 were able to inhibit cAMP production in response to [D-Trp^8]-SRIF-14. However, these cells did not inhibit cAMP production as robustly in response to L-779,976. These results provide functional evidence to support our claim that a proportion of the cell surface binding sites maintained following 40 min of L-779,976 stimulation were sst_5 receptors.

Our confocal images suggest that in the absence of agonist, the distribution of sst_5 is primarily intracellular. However, perinuclear accumulation following stimulation with [D-Trp^8]-SRIF-14 indicates that agonist-induced internalization of sst_5 receptors occurs in these cell lines, which suggests that at least some sst_5 is localized at the cell surface under baseline conditions. In addition, based on our previous findings in COS-7 cells (Stroh *et al.*, 2000b), it is tempting to speculate that sst_5 receptors are targeted to the cell surface in response to agonist stimulation. However, we have provided no direct evidence for this in the present study so we can only hypothesize as to the regulation of sst_5 receptors.

By characterizing the internalization and trafficking events induced following selective stimulation of sst_{2A}, we have provided evidence that sst_5 influences these regulatory processes. Specifically, we have demonstrated that while stimulation of sst_{2A} is sufficient to induce internalization, trafficking and recycling of the receptor when it is expressed alone, dual stimulation of sst_{2A} and sst_5 is required for these events to take place when the receptors are co-expressed. Therefore, our findings suggest a functional association between sst_{2A} and sst_5 receptors, and fit well with recent reports suggesting that these receptor subtypes interact to modulate the cellular effects of SRIF (Ren *et al.*, 2003; Cervia *et al.*, 2003c; Ben-Shlomo et al., 2005). Furthermore, we have demonstrated for the first time that cellular desensitization following stimulation with a

sst_2-selective SRIF analog is attenuated by the presence of sst_5 receptors. This suggests that the mechanisms underlying cellular desensitization in cells that express both sst_{2A} and sst_5 are mediated through the combined presence of these receptor subtypes.

While the development of tolerance to pharmacological agents is a very complex condition that involves several stages of physiological adaptation, agonist-induced changes in receptor trafficking are thought to play a role for drugs targeting other neuropeptide receptors such as the opioids (von Zastrow, 2004). Therefore, the absence of tolerance to drugs targeting SRIF receptors may be partially due to specific regulatory mechanisms occurring at the level of the cell. Furthermore, one could speculate that the regulatory events that we have described might account for the absence of tolerance to pharmacological agents that are prescribed for the treatment of pituitary adenomas. Specifically, since Octreotide® and other octapeptide SRIF analogs preferentially bind to sst_2 receptors, it is conceivable that cellular responsiveness to these SRIF receptor agonists is maintained through the phenomenon that we have characterized.

Taken together, our results indicate that the regulation of sst_{2A} and sst_5 receptors is interconnected. Although the precise mechanism of the hypothesized sst_{2A}-sst_5 interaction is unknown, our preliminary findings suggest that there is no physical interaction between these receptor subtypes. While several recent studies have shown that SRIF receptors

homo- and heterodimerize (Rocheville *et al.*, 2000; Pfeiffer *et al.*, 2001; Patel *et al.*, 2002; Grant *et al.*, 2004a, 2004b), to date there is no evidence that sst_{2A} and sst_5 form heterodimers. Indeed, our immunocytochemical evidence suggests that these receptor subtypes do not co-localize upon agonist stimulation. However, agonist stimulation has been demonstrated to regulate the formation of sst_5 homodimers (Rocheville *et al.*, 2000; Patel *et al.*, 2002). Furthermore, sst_2 homodimers have been demonstrated to exist at the cell surface in the absence of ligand (Pfeiffer *et al.*, 2001; Grant *et al.*, 2004a), and SRIF-14-induced dissociation of homodimers has been shown to be necessary for sst_2 internalization (Grant *et al.*, 2004a). Therefore, while current knowledge suggests that the hypothesized sst_{2A}-sst_5 interaction is not mediated through the formation of heterodimers, the formation and/or dissociation of sst_{2A} or sst_5 homodimers may influence this process and affect the cellular regulation of these receptor subtypes.

A Proposed Model for the Cellular Regulation of Sst_{2A} and Sst_5 Receptors in Cells Expressing Both Receptor Subtypes

In the present Master's thesis, we have characterized a novel phenomenon of agonist-induced sst_{2A} regulation observed in cells that co-express sst_{2A} and sst_5. However, the underlying cellular and molecular mechanisms mediating our observations have not yet been delineated. Therefore, based on our experimental work, the current literature on SRIF receptor biology and hypothetical mechanisms, we have developed a

model to help explain our observations in cells expressing both sst_{2A} and sst_5 receptors (see Fig. 11).

Upon stimulation with [D-Trp^8]-SRIF-14 (Fig. 11*A*), cell surface sst_{2A} receptors rapidly internalize (*step 1*) and accumulate in a perinuclear compartment identified as the TGN-pericentriolar recycling endosome (*step 2*). The recovery of cell surface sst_{2A} receptors occurs in the absence of agonist (*step 3*), in a process that is sensitive to monensin, indicating that recycling involves early endosomes. Co-stimulation of sst_5 by [D-Trp^8]-SRIF-14 induces internalization and perinuclear accumulation of the receptor (*step 4*), and possibly targeting of spare receptors to the cell surface (*step 5*). Furthermore, we propose that the activation of sst_5 receptors initiates a sst_{2A}-directed signal (*step 6*), which promotes the internalization, intracellular trafficking and recycling sst_{2A}. To our knowledge, no such signaling pathway between SRIF receptor subtypes has yet been characterized.

Stimulation with L-779,976 (Fig. 11*B*) induces a completely different pattern of sst_{2A} internalization and trafficking as compared with [D-Trp^8]-SRIF-14 stimulation. Since sst_5 is not activated by L-779,976, our hypothesized sst_{2A}-directed signal is not initiated (*step 1*). Our findings indicate that L-779,976 induces internalization of sst_{2A} receptors, but that sst_{2A} remains localized at the cell periphery following stimulation with this agonist. Furthermore, the recovery of cell surface sst_{2A} does not occur in the absence of agonist. Accordingly, monensin did not effect cell surface

sst_{2A} receptor density. Therefore, we hypothesize that following stimulation with L-779,976, cell surface sst_{2A} receptors are either poorly internalized or are immediately recycled back to the cell surface (*step 2*). The overall result of these trafficking events is a relative maintenance of cell surface receptor density, and thus continued responsiveness to agonist stimulation.

Conclusion

In this Master's thesis, we have characterized the agonist-induced trafficking of sst_{2A} and sst_5 receptors in epithelial cell lines that ectopically express these receptor subtypes and a pituitary cell line that expresses sst_{2A} and sst_5 endogenously. Furthermore, we have provided evidence for a novel phenomenon mediating the regulation of sst_{2A} receptors. In cells expressing both receptor subtypes, we have shown that selective stimulation of sst_{2A} reduces cell surface receptor density to a lesser extent than in cells expressing sst_{2A} alone. Taken together, our findings suggest that sst_5 influences the internalization, intracellular trafficking and signaling of sst_{2A} receptors, and that the combined activity of these receptor subtypes attenuates cellular desensitization to SRIF agonists following continuous stimulation.

Based on our results, we conclude that the cellular regulation of sst_{2A} and sst_5 receptors is interconnected. While the precise mechanism of this interaction remains to be elucidated, our findings contribute to the growing evidence suggesting that sst_{2A} and sst_5 interact to modulate the inhibitory effects of SRIF. Furthermore, we have provided evidence to suggest that maintenance of responsiveness to SRIF analogs can be observed at the cellular level, and that the mechanisms mediating this process occur through the combined activity of sst_{2A} and sst_5 receptors. Therefore, our work has not only contributed to the understanding of the cellular regulation of sst_{2A} and sst_5 receptors, but has provided cellular

models that offer important insights into the mechanisms whereby SRIF analogs targeting pituitary adenomas are able to mediate their effects without the development of tolerance.

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Appendix

Research Compliance Certificates (next 2 pages):

- Radiation safety certification (1 page)
- Internal radioisotope users permit (1 page)