Agonist-dependent Up-regulation of Human Somatostatin Receptor Type 1 Requires Molecular Signals in the Cytoplasmic C-tail*

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We have previously reported that the human somatostatin receptor type 1 (hSSTR1) stably expressed in Chinese hamster ovary-K1 cells does not internalize but instead up-regulates at the membrane during continued agonist treatment (1 μM somatostatin (SST)-14 × 22 h). Here we have investigated the molecular basis of hSSTR1 up-regulation. hSSTR1 was up-regulated by SST in a time-, temperature-, and dose-dependent manner to saturable levels, in intact cells but not in membrane preparations. Although hSSTR1 was acutely desensitized to adenyl cyclase coupling after 1 h SST-14 treatment, continued agonist exposure (22 h) restored functional effector coupling. Up-regulation was unaffected by cycloheximide but blocked by okadaic acid. Confocal fluorescence immunocytochemistry of intact and permeabilized cells showed progressive, time-dependent increase in surface hSSTR1 labeling, associated with depletion of intracellular SSTR1 immunofluorescently labeled vesicles. To investigate the structural domains of hSSTR1 responsible for up-regulation, we constructed C-tail deletion (Δ) mutants and chimeric hSSTR1-hSSTR5 receptors. Human SSTR5 was chosen because it internalizes readily, displays potent C-tail internalization signals, and does not up-regulate. Like wild type hSSTR1, Δ C-tail hSSTR1 did not internalize and additionally lost the ability to up-regulate. Swapping the C-tail of hSSTR1 with that of hSSTR5 induced internalization (27%) but not up-regulation. Substitution of hSSTR5 C-tail with that of hSSTR1 converted the chimeric receptor to one resembling wild type hSSTR1 (poor internalization, 71% up-regulation). These results show that ligand-induced up-regulation of hSSTR1 occurs by a temperature-dependent active process of receptor recruitment from a pre-existing cytoplasmic pool to the plasma membrane. It does not require new protein synthesis or signal transduction, is sensitive to dephosphorylation events, and critically dependent on molecular signals in the receptor C-tail.

Somatostatin (SST), a naturally occurring regulatory peptide with two biologically active forms, SST-14 and SST-28, is produced in neural, endocrine, and immune cells and exerts potent effects on many different tissue targets including the brain, pituitary, pancreas, gut, thyroid, adrenals, and kidneys (1–3). The cellular actions of SST include the inhibition of hormone and exocrine secretion as well as modulation of neurotransmission and cell proliferation and are mediated by G protein-coupled receptors (GPCR) (2, 3). Somatostatin receptors (SSTR) belong to the family of seven transmembrane domain proteins and comprise five distinct subtypes that are encoded by separate genes (2, 3). In the case of the human receptors, four of the isoforms (hSSTR1–4) display weak selectivity for binding to SST-14, whereas hSSTR5 shows preference for SST-28 binding (2). SSTRs are widely expressed in many tissues frequently as multiple subtypes that coexist in the same cell (2–6). The five receptors share common signaling pathways such as the inhibition of adenyl cyclase, activation of phosphotyrosine phosphatase, or modulation of mitogen-activated protein (MAP) kinase through G protein-dependent mechanisms (2, 3, 7). Some of the subtypes are also coupled to K+ and Ca2+ ion channels, to phospholipase C, and phospholipase A2 (2). hSSTR1 activates a Na+/H+ exchanger via a non-G protein-linked pathway (8).

A common property of most GPCRs is their ability to regulate their responsiveness to continued agonist exposure (9). Such agonist-specific regulation typically involves receptor desensitization due to uncoupling from G proteins, as well as receptor internalization and receptor degradation (9). The underlying molecular mechanisms have been extensively studied in the case of the β-adrenergic and several other GPCRs, and a general model has been proposed that involves phosphorylation of the C-tail and intracellular loops of the agonist-occupied receptor by a second messenger activated or G protein-coupled receptor kinase, resulting in rapid attenuation of receptor signaling. G protein-coupled receptor kinase phosphorylation promotes the binding of β-arrestin, which acts as an adapter molecule linking the receptor to clathrin-mediated endocytosis (9–11). The endocytosed receptor is either sorted to lysosomes for degradation if agonist stimulation is prolonged or recycled back to the cell surface as a result of pH-dependent conformational change and dephosphorylation by a membrane-associated GPCR phosphatase in endosomes (9–11). Another less
well known type of agonist-induced receptor regulation is the property of receptor up-regulation that occurs in response to chronic agonist stimulation of receptors such as the β3-adrenergic receptor (β3AR) (12), 5HT2A (13, 14), gonadotropin-releasing hormone (GnRH) (15), angiotensin II (16), dopamine 3 (17), the long form of dopamine 2 (D2LR) (18–21), and endogenous SSTRs (22). Since continuous exposure of receptors to agonists is unlikely to occur under normal physiological conditions, this type of response appears to be pharmacological and is observed during long term drug therapy or in disease states. Unlike receptor down-regulation, the underlying molecular mechanisms for up-regulation are poorly understood and thought to involve ligand-induced transcriptional or posttranscriptional induction of receptor synthesis and targeting to the plasma membrane (12–20).

In the case of the SSTR family, we have previously reported that hSSTR5 stably expressed in CHO-K1 cells undergoes rapid agonist-dependent desensitization and internalization, whereas hSSTR1 under the same conditions fails to be internalized and is up-regulated at the plasma membrane following prolonged agonist exposure (23, 24). To identify the underlying molecular signals, we have here created C-tail deletion mutants and hSSTR1/hSSTR5 chimeras, and we have analyzed the ability of these mutant receptors to undergo agonist-dependent internalization or up-regulation as well as G protein-linked coupling to adenyl cyclase. We have further investigated membrane and intracellular trafficking of hSSTR1 as well as the relationship between the internalization and up-regulation pathways. We report that the up-regulated receptor is functionally coupled to G proteins and that up-regulation of hSSTR1 is an intrinsic property of the receptor that occurs in the absence of endocytosis or new protein synthesis by an active process of receptor recruitment from the cytoplasm to the cell surface. Furthermore, the receptor C-tail contains molecular signals that specify up-regulation.

**EXPERIMENTAL PROCEDURES**

**Materials**—SST-14, SST-28, and Leu°, n-Tyr°, SST-28 (LTT SST-28) were from Bachem (Marina Del Rey, CA). Cycloheximide, pertussis toxin, okadaic acid, phenylmethylsulfonyl fluoride, and bacitracin were from Sigma. Carrier-free Na125I was obtained from Amer sham Pharmacia Biotech. Rhodamine-conjugated goat anti-rabbit IgG was from Jackson Immunoresearch Laboratories (West Grove, PA). Ham’s F-12 medium, fetal bovine serum, and G418 were from Life Technologies Inc. Cyclic AMP radioimmunoassay kits were obtained from Diagnostic Products Corp. (Los Angeles, CA). All other reagents were of analytical grade and purchased from various suppliers.

**Construction of Wild Type, Mutant, and Chimeric Receptors**—cDNA for wild type hSSTR5 was created as a cassette construct in PTEJ8. Wild type (wt) hSSTR1 DNA encoding the complete receptor sequence was generated by PCR amplification using human genomic DNA as template and subcloned into the pDNA3 expression vector. A series of
mutant and chimeric hSSTR1/hSSTR5 receptors were created to investigate the role of the C-tail in the internalization and up-regulation properties of the receptors (Fig. 1). The cytoplasmic tail (C-tail) of wt hSSTR1 contains 65 amino acid residues with 5 tyrosine residues and 11 serine or threonine residues that could serve as putative phosphorylation sites. The wt hSSTR5 C-tail contains 55 amino acid residues with 7 potential serine/threonine phosphorylation sites. C-tail deletions were created at position 318 for hSSTR5 (Δ C-tail hSSTR5) and at position 331 for hSSTR1 (Δ C-tail hSSTR1). Chimeric receptors were constructed by fusing the C-tail of hSSTR5 with the C-tail of hSSTR1 and of the C-tail of hSSTR1 with the C-tail of hSSTR5. Mutations were created by the PCR overlap extension technique (25); for the C-tail-truncated mutants, oligonucleotide primers were used that contain an appropriately placed stop codon after position 331 (hSSTR1) and position 318 (hSSTR5). Chimeric receptors were constructed using oligonucleotide primers designed to allow separate amplification of 5′, 3′, and internal segments that were subsequently fused by PCR. All primers were designed so as to avoid any change in the reading frame as follows: primer A, 5′-GATCAATTCTTATCAGAGCGTCGTGATCCGG-3′ (R1 forward); primer B, 5′-CAGGCCCCGCGCCCACACACCCCCAGGATAGAT-3′ (R1 reverse); primer C, 5′-TGCGGCCGGGCCGCTG-3′ (R1 forward); primer D, 5′-GCACGTTTCTTACGCAGAACC-3′ (R1 reverse); primer E, 5′-GATAAGCTTCTTGAGGAGTAGAT-3′ (R1 reverse); primer F, 5′-TCGGGCGTGGTGATGTTCCAGG-3′ (R1-R5 forward); primer G, 5′-TCGTGAACTTTCCTCAAAGCGCCTC-3′ (R5-R1 forward); primer H, 5′-GATCAGATTCTTATCAGAGCGTCGTGATCCGG-3′ (R1 reverse).

To construct the Δ C-tail hSSTR1/C-tail hSSTR5 chimera, primer pairs A and B were used to synthesize the 5′ fragment of hSSTR1 using SSTR1 cDNA as template for PCR. Primer pairs C and D were used to generate the 3′ C-terminal fragment using SSTR5 cDNA as template. PCR was carried out with 50 ng of SSTR cDNA in 100 μl containing 20 mM Tris-HCl, pH 8.5, 50 mM KCl, 200 μM dNTPs, 1.5 mM MgCl₂, 7% Me₂SO, and 2.00 units of Pfu polymerase (Stratagene). The PCR conditions were as follows: denaturation at 94 °C for 1 min, annealing at 58 °C for 50 s, and extension at 72 °C for 25 cycles followed by extension at 72 °C for 10 min. PCR products were separated by agarose gel electrophoresis; the amplified bands were electroeluted and purified. Receptor fragments A—B and C—D were then fused in a third PCR reaction to generate the full-length chimeric receptor in a ligation reaction using flanking primer pairs A and D. The Δ C-tail hSSTR5 + C-tail hSSTR1 chimera was generated using primer pairs E—F and G—H to synthesize the 5′ and 3′ receptor fragment sequences, respectively. The purified amplification products were ligated by PCR using primer pairs E and H. All 5′-flanking primers contained HindIII endonuclease restriction sites, Kozak consensus sequences, and initiation codons. All 3′-flanking primers comprised a stop codon followed by an EcoRI restriction site. After PCR ligation, the products were digested to completion with HindIII and EcoRI, and purified fragments were subcloned into the HindIII-EcoRI multiple cloning sites of pTEJ8. The structure of all mutant and chimeric receptor constructs was confirmed by sequence analysis (University Core DNA Service, University of Calgary, Alberta, Canada). CHO-K1 cells were transfected with cDNAs for wild type or mutant and chimeric hSSTR1/hSSTR5 receptors by the Lipofectin method (Life Technology, Inc.), and stable G418-resistant nonclonally selected cells were propagated for study. wt hSSTR1 was also stably expressed in HEK-293 cells by the same method.

**Binding Assays—**CHO-K1 cells expressing wild type, mutant, and chimeric receptors were cultured in 6-well plates in F-12 medium without fetal calf serum with 20–40 μg of membrane protein and [125I]-LTT SST-28 radioligand in 50 mM Heps, pH 7.5, 2 mM CaCl₂, 5 mM MgCl₂, 0.5% bovine serum albumin, 0.02% phenylmethylsulfonyl fluoride, and 0.02% bacitracin (binding buffer) as described previously (23, 24). Saturation binding experiments were performed with membranes using increasing concentrations of [125I]-LTT SST-28 (2–2000 pM) under equilibrium binding conditions (24). Incubations were terminated by the addition of 1 ml of ice-cold phosphate-buffered saline containing 0.2% bovine serum albumin, rapid centrifugation, and washing. Radioactivity associated with membrane pellets was quantified in an LKB gamma counter (LKB-Wallach, Turku, Finland). Binding data were analyzed with INPLOT 4.03 (Graph Pad Software, San Diego, CA). The binding assay was tested by incubating cells for 30 min with 1 μM forskolin and 0.5 μM 3-isobutyl-1-methylxanthine with or without SST (10⁻⁶ - 10⁻¹¹ M) at 37 °C as described previously (24). Cells were then scraped in 0.1 N HCl and assayed for CAMP by radioimmunoassay.

**Internalization Experiments (Acute Agonist Exposure)—**CHO-K1 cells expressing wild type, mutant, and chimeric SSTRs were cultured in 6-well plates and studied at ~90% confluency (1.5 × 10⁸ cells/well). Cells were equilibrated overnight at 4 °C with [125I]-LTT SST-28 with or without 100 nM SST-14 (for hSSTR1) or 100 nM SST-28 (for hSSTR5). After washing, cells were warmed to 37 °C for 15, 30, and 60 min to initiate internalization (23, 24). At the end of each incubation, surface-bound radioligand was removed by treatment for 10 min at 37 °C with 1 ml of acid wash (Hanks'-buffered saline acidified to pH 5.0 with 20 μM sodium acetate). Internalized radioligand was measured as acid-resistant counts in 0.1 N NaOH extracts of acid-washed cells.

**Up-regulation Experiments (Chronic Agonist Exposure)—**CHO-K1 cells expressing wild type, mutant, and chimeric receptors were cultured in 6-well plates in F-12 medium without fetal calf serum with 10⁻⁷ M SST-14 or SST-28 for 4, 9, 13, 16, 19, and 22 h at 37 °C. After acid wash to remove surface-bound SST, whole cell binding assays were performed to determine total and nonspecific binding (24). Residual surface binding was calculated as the difference between control and experimental groups. Dose dependence of up-regulation was studied by incubating cells with 10⁻¹¹–10⁻⁶ M SST-14 for 22 h at 37 °C. The effect of blocking protein synthesis on up-regulation of hSSTR1 was investigated by applying cycloheximide 10 μg/ml for 30 min to CHO-K1 cells expressing wt hSSTR1 as described previously (22). The effect of pertussis toxin and okadaic acid on SST-induced hSSTR1 up-regulation was determined by continuous treatment with pertussis toxin 100 ng/ml or okadaic acid 200 nM followed by whole cell binding analyses. To investigate the fate of the up-regulated membrane receptor, cells were cultured for 22 h with 10⁻⁷ M SST-14 to induce up-regulation. Cells were then washed gently with 1 ml of 20 mM sodium acetate pH 5.0 for 2 min to remove surface-bound SST-14 and reincubated in culture medium without ligand. Residual surface receptors were analyzed at 12 and 22 h by whole cell binding.

**Immunocytochemistry—**To analyze surface and cytoplasmic pools of receptors, intact or 0.2% Triton X-100-permeabilized CHO-K1 cells expressing wt hSSTR1 or wt hSSTR5 were processed for confocal fluorescence immunochemistry using rabbit polyclonal antipeptide receptor antibodies (4–6, 24). CHO-K1 cells expressing SSTRs were cultured to ~70% confluency and treated with SST for different times. To analyze surface expression of receptors, cells were incubated in serum-free Ham's F-12 medium supplemented with 1% bovine serum albumin in the presence of SST primary antibodies for 8–12 h at 4 °C. After washing in 50 mM Tris-HCl, 0.9% NaCl (TBS), pH 7.4, cells were fixed for 30 min at 4 °C in 4% paraformaldehyde. To label the cytoplasmic pool of receptors, cells were permeabilized with 0.2% Triton X-100 in TBS for 5 min at room temperature, washed three times in TBS, and incubated with SST primary antibodies for 8–12 h at 4 °C. Antipeptide antibodies directed against the N-terminal segment of hSSTR1
Determined by whole cell binding with 125I-LTT-SST-28 ligand. After 60 min at 37 °C, whereas hSSTR1 under comparable temperature-dependent manner with 66% bound to hSSTR5 was rapidly internalized in a time- and 6-fold higher concentration at 4 °C (Fig. 2A). Up-regulation of hSSTR1 was temperature-dependent and was reduced to 44 ± 16% at 20 °C and virtually abolished at 4 °C (Fig. 2A). Up-regulation was also dose-dependent over the concentration range 10^{-11}–10^{-6} M SST-14 (Fig. 2C). When cells expressing hSSTR1 were first treated with SST-14 (10^{-7} M) for 22 h to up-regulate the receptors, and the SST was then removed, there was a slow loss of surface hSSTR1 expression from 110% at time 0 to 48 ± 9% at 12 h and 8 ± 3% at 22 h (Fig. 2B). To determine whether receptor up-regulation was a membrane phenomenon due to aggregation or clustering, membranes rather than whole cells were pre-exposed to SST-14 for 22 h at 37 °C in binding buffer with protease inhibitors (Protease Inhibitor Mixture, 1 tablet/50 ml binding buffer, Roche Molecular Biochemicals). Under these conditions, receptor concentration (B_{max}) immediately after the preparation of membranes was 229 ± 10 fmol/mg protein and did not change significantly when incubated in binding buffer alone for 22 h at 37 °C indicating stability of the receptor in the membrane preparation. In contrast to whole cells, however, hSSTR1 in membrane preparations showed no up-regulation during 22 h treatment with 10^{-7} M SST-14. This suggests that up-regulation is a temperature-dependent, active process requiring the intact cell. Treatment of hSSTR1 cells with pertussis toxin reduced up-regulation by 36 ± 8% suggesting that the up-regulation response is only partly mediated by G_i or G_o proteins (Fig. 3). Pretreatment of cells with cycloheximide (10 μg/ml for 30 min) had no effect on hSSTR1 up-regulation, whereas okadaic acid completely abolishes the up-regulation response. Okadaic acid (200 nM) completely abolished up-regulation. This suggests that up-regulation of hSSTR1 does not require new protein synthesis but is dependent on dephosphorylation events. To exclude the possibility that up-regulation is a peculiarity of CHO-K1 cell transfection or the level of receptor expression, HEK-293 cells transfected with hSSTR1 were analyzed. These cells expressed 5-fold higher density of hSSTR1 (B_{max} 1.2 ± 0.135 pmol/mg protein); like CHO-K1 cells they failed to internalize 125I-LTT SST-28 and displayed 97 ± 16% up-regulation of cell surface binding after continuous treatment with SST-14 (10^{-7} M) for 22 h at 37 °C.

**Fig. 2. Effect of time, temperature, and agonist (SST-14) concentration on surface hSSTR1 expression in stable CHO-K1 cells determined by whole cell binding with 125I-LTT-SST-28 ligand.** A. 10^{-7} M SST induces surface-receptor expression in a time- and temperature-dependent manner. , 37 °C; ●, 20 °C; ○, 4 °C. B, removal of SST-14 results in a slow loss of surface hSSTR1-binding sites over 48 h. C, agonist dose-response curve of hSSTR1 up-regulation. (Mean ± S.E. of three independent experiments in triplicate.)

**Results**

**Binding Characteristics of C-tail Deletion and Chimeric Receptors**—The C-tail deletion and chimeric receptors were correctly targeted to the plasma membrane as determined by binding analysis (Table I). Saturation binding analysis of CHO-K1 cell membranes revealed a comparable level of expression of wt hSSTR1 and wt hSSTR5 (229 ± 10 and 180 ± 28 fmol/mg protein, respectively). The Δ C-tail hSSTR1 mutant displayed a small reduction in B_{max} (113 ± 14 fmol/mg) and binding affinity (K_d 2.3 nM compared with K_d 0.62 nM for wt hSSTR1). As previously reported, the Δ C-tail hSSTR5 mutant displayed high affinity ligand binding (K_d 0.89 nM) which, however, was 3-fold lower than that of the wild type receptor (21). In contrast, the binding parameters of the Δ C-tail hSSTR5 mutant were comparable to those of the wild type receptor. Likewise, the K_d and B_{max} of the two chimeric receptors were comparable to that of wt hSSTR1 and wt hSSTR5.

**Agonist-induced Regulation of wt hSSTR1 and wt hSSTR5**—As reported previously, 125I-LTT SST-28 when bound to hSSTR5 was rapidly internalized in a time- and temperature-dependent manner with 66 ± 7% internalization after 60 min at 37 °C, whereas hSSTR1 under comparable incubation conditions showed no internalization (21, 22). Furthermore, long term exposure to SST-14 or SST-28 (10^{-7} M) induced time-dependent up-regulation of hSSTR1 (110 ± 17% increase in surface binding after 22 h at 37 °C) with no effect on hSSTR5 (Fig. 2A). Up-regulation of hSSTR1 was temperature-dependent and was reduced to 44 ± 16% at 20 °C and virtually abolished at 4 °C (Fig. 2A). Up-regulation was also dose-dependent over the concentration range 10^{-11}–10^{-6} M SST-14 (Fig. 2C). When cells expressing hSSTR1 were first treated with SST-14 (10^{-7} M) for 22 h to up-regulate the receptors, and the SST was then removed, there was a slow loss of surface hSSTR1 expression from 110% at time 0 to 48 ± 9% at 12 h and 8 ± 3% at 22 h (Fig. 2B). To determine whether receptor up-regulation was a membrane phenomenon due to aggregation or clustering, membranes rather than whole cells were pre-exposed to SST-14 for 22 h at 37 °C in binding buffer with protease inhibitors (Protease Inhibitor Mixture, 1 tablet/50 ml binding buffer, Roche Molecular Biochemicals). Under these conditions, receptor concentration (B_{max}) immediately after the preparation of membranes was 229 ± 10 fmol/mg protein and did not change significantly when incubated in binding buffer alone for 22 h at 37 °C indicating stability of the receptor in the membrane preparation. In contrast to whole cells, however, hSSTR1 in membrane preparations showed no up-regulation during 22 h treatment with 10^{-7} M SST-14. This suggests that up-regulation is a temperature-dependent, active process requiring the intact cell. Treatment of hSSTR1 cells with pertussis toxin reduced up-regulation by 36 ± 8% suggesting that the up-regulation response is only partly mediated by G_i or G_o proteins (Fig. 3). Pretreatment of cells with cycloheximide (10 μg/ml for 30 min) had no effect on hSSTR1 up-regulation, whereas okadaic acid completely abolishes the up-regulation response. Okadaic acid (200 nM) completely abolished up-regulation. This suggests that up-regulation of hSSTR1 does not require new protein synthesis but is dependent on dephosphorylation events. To exclude the possibility that up-regulation is a peculiarity of CHO-K1 cell transfection or the level of receptor expression, HEK-293 cells transfected with hSSTR1 were analyzed. These cells expressed 5-fold higher density of hSSTR1 (B_{max} 1.2 ± 0.135 pmol/mg protein); like CHO-K1 cells they failed to internalize 125I-LTT SST-28 and displayed 97 ± 16% up-regulation of cell surface binding after continuous treatment with SST-14 (10^{-7} M) for 22 h at 37 °C.
Confocal Fluorescence Immunocytochemistry of hSSTR1 and hSSTR5—In this experiment, we investigated changes in the pattern of expression of hSSTR1 and hSSTR5 proteins in the plasma membrane and intracellular compartments by immunofluorescence with antipeptide receptor antibodies in intact and permeabilized CHO-K1 cells stably expressing hSSTR1 and hSSTR5 (Figs. 4 and 5). After treatment with SST-14 for 0, 1, 16, and 22 h, nonpermeabilized hSSTR1 cells (Fig. 4, A, C, E, and G) displayed surface labeling which increased progressively with SST-14 treatment. Permeabilized cells reveal labeling of ill-defined small cytoplasmic vesicular structures at 0 and 1 h which decrease over time with agonist treatment. Scale bar, 25 μm.

Fig. 4. Immunoconfocal optical sections illustrating fluorescence analysis of hSSTR1 in stably transfected CHO-K1 cells. After treatment with SST-14 for 0, 1, 16, and 22 h, nonpermeabilized cells (A, C, E, and G) and Triton X-100-permeabilized cells (B, D, F, and H) were labeled with rabbit anti-hSSTR1 primary antibody and rhodamine-conjugated goat anti-rabbit secondary antibody. Nonpermeabilized hSSTR1 cells display surface labeling which increases progressively with SST-14 treatment. Permeabilized cells reveal labeling of ill-defined small cytoplasmic vesicular structures at 0 and 1 h which decrease over time with agonist treatment. Scale bar, 25 μm.

Time Course of Agonist Pretreatment on hSSTR1 Coupling to Adenyl Cyclase—To determine the effect of continued agonist exposure on the desensitization response, we investigated coupling of hSSTR1 to adenyl cyclase after 0, 1, and 22 h pretreatment with SST-14 (10^{-7} M). After removal of surface-bound SST-14 by acid wash, the ability of subsequently added agonist to inhibit forskolin-stimulated cAMP accumulation was determined (Fig. 6). Control cells (time 0) displayed dose-dependent maximum 68 ± 4% inhibition of forskolin-stimulated cAMP with 10^{-6} M SST-14. One hour pretreatment with

FIG. 5. Immunoconfocal optical sections illustrating immunofluorescence analysis of hSSTR5 in stably transfected CHO-K1 cells. Cells were treated with SST-14 for 0, 1, 16, and 22 h, and receptor immunoreactivity was detected in intact (A, C, E, and G) and permeabilized (B, D, F, and H) cells by immunofluorescence using rabbit anti-hSSTR5 primary antibody and rhodamine-conjugated goat anti-rabbit secondary antibody. Permeabilized hSSTR5 cells show a well defined population of hSSTR5-positive cytoplasmic vesicles which remain the same in density during continued agonist exposure. Scale bar, 25 μm.

Fig. 5. Immunoconfocal optical sections illustrating immunofluorescence analysis of hSSTR5 in stably transfected CHO-K1 cells. Cells were treated with SST-14 for 0, 1, 16, and 22 h, and receptor immunoreactivity was detected in intact (A, C, E, and G) and permeabilized (B, D, F, and H) cells by immunofluorescence using rabbit anti-hSSTR5 primary antibody and rhodamine-conjugated goat anti-rabbit secondary antibody. Permeabilized hSSTR5 cells show a well defined population of hSSTR5-positive cytoplasmic vesicles which remain the same in density during continued agonist exposure. Scale bar, 25 μm.

FIG. 6. Time course of agonist pretreatment on hSSTR1 coupling to adenyl cyclase. Control cells display dose-dependent maximum 68% inhibition of forskolin-stimulated cAMP with 10^{-6} M SST-14 which is reduced to 29% after 1 h pretreatment with SST-14 (10^{-7} M) suggesting receptor uncoupling (●). After 22 h pretreatment with SST-14, receptor coupling to adenyl cyclase is partially restored (●) suggesting that the up-regulated membrane receptors are not desensitized but are functionally coupled to G protein-linked effector pathways (mean ± S.E. of three independent experiments in triplicate).

SST-14 to inhibit forskolin-stimulated cAMP accumulation was determined (Fig. 6). Control cells (time 0) displayed dose-dependent maximum 68 ± 4% inhibition of forskolin-stimulated cAMP with 10^{-6} M SST-14. One hour pretreatment with
SST-14 markedly reduced the inhibitory effect of SST-14 on forskolin-stimulated cAMP to a maximum of 29 ± 3.4% suggesting receptor uncoupling. After 22 h pretreatment with SST-14, absolute cAMP levels increased 2.4-fold. At the same time, the maximum forskolin-stimulated cAMP inhibition by SST-14 increased to 45 ± 4.1% indicating partial restoration of receptor coupling to adenyl cyclase. These results suggest that the up-regulated membrane receptors are not desensitized but are functionally coupled to G protein-linked effector pathways.

**Internalization of Mutant and hSSTR1-hSSTR5 Chimeric Receptors**—Fig. 7 and Table I depict the internalization profiles of 125I-LT SST-28 incubated over 60 min with CHO-K1 cells expressing C-tail deletion mutants and hSSTR1-hSSTR5 chimeric receptors. Compared with internalization of wt hSSTR5 (66 ± 7% at 60 min), truncation of the C-tail reduced internalization to 44 ± 5%. In the case of hSSTR1, both the wild type and C-tail deletion mutants displayed a comparable inability to undergo agonist-promoted endocytosis. Replacement of hSSTR5 C-tail with the C-tail of hSSTR1 completely abolished internalization of the chimeric receptor. This suggests the presence of potent negative internalization signals in the C-tail of hSSTR1 sufficient to block internalization of hSSTR5. The C-tail signals alone, however, cannot explain the inability of hSSTR1 to internalize, since deletion of hSSTR1 C-tail did not induce internalization suggesting the additional involvement of other intracellular domains. Replacement of the hSSTR1 C-tail with the C-tail of hSSTR5 induced 27 ± 9% internalization confirming the presence of internalization signals in hSSTR5 C-tail (24).

**Up-regulation of Mutant and hSSTR1-hSSTR5 Chimeric Receptors**—Fig. 8 and Table I illustrate the results of whole cell binding analysis of mutant and chimeric receptors treated with SST-14 for 22 h. Like wt hSSTR5, the Δ C-tail hSSTR5 mutant showed no agonist-dependent increase in cell surface binding. Deletion of the C-tail of hSSTR1, however, completely abolished the ability of this receptor to undergo agonist-dependent up-regulation. The chimeric Δ C-tail hSSTR1 + C-tail hSSTR5 receptor behaved identically to the Δ C-tail hSSTR1 receptor in showing a complete absence of up-regulation. Substitution of hSSTR5 C-tail with that of hSSTR1, however, converted the chimeric receptor to one resembling wt hSSTR1 with 71 ± 18% up-regulation at the cell surface. This suggests that up-regulation is a functional property of hSSTR1 and is dependent on molecular signals localized in the receptor C-tail.

**Coupling of Mutant and Chimeric hSSTR1-hSSTR5 Receptors to Adenyl Cyclase**—To determine the influence of receptor signaling capability, if any, on the up-regulation process, we determined the ability of mutant and chimeric hSSTR1-hSSTR5 receptors to inhibit forskolin-stimulated cAMP by SST-14 (Fig. 9 and Table I). Deletion of the hSSTR1 C-tail reduced its ability to inhibit forskolin-stimulated cAMP by 23% (from 68 ± 4 to 46 ± 3%). In contrast, as previously shown, deletion of the C-tail of hSSTR5 completely abolished the ability of this receptor to couple to adenyl cyclase. The two C-tail chimeric constructs maintained some ability to inhibit forskolin-stimulated cAMP; the maximum inhibitory response, however, was reduced to 38 ± 4 and 30 ± 3% for the Δ C-tail hSSTR1 + C-tail hSSTR5 and Δ C-tail hSSTR5 + C-tail hSSTR1 chimeras, respectively.

**DISCUSSION**

Although negative regulation by agonists has been established as a fundamental property of most GPCRs (reviewed in Ref. 9), there are only sporadic reports describing the opposite phenomenon of receptor up-regulation by agonists (12–23). This is because unlike acute receptor desensitization, which is clearly a physiological event, receptor up-regulation is elicited only during prolonged agonist stimulation and is consequently less well characterized. Agonist-induced up-regulation has been shown not only for GPCRs but applies to other classes of membrane proteins as well, such as the nicotinic acetylcholine receptor and may, therefore, be a fundamental cellular response (26). Up-regulated receptor function may explain drug tolerance and the ability of receptors such as the D2 receptor and SSTRs to maintain normal responsiveness during long term pharmacotherapy (2, 3, 21). Several different mechanisms have been described. In cultured rat pituitary cells, GnRH up-regulates its receptor after a delay of 6 h by a process that is dependent on extracellular Ca2+ and new protein synthesis (15). Agonist-mediated up-regulation of 5HT2A receptor in cerebellar granule neurons requires transcriptional induction of receptor mRNA by receptor-activated Ca2+ influx and activation of calmodulin kinase (13, 14). Likewise, the β,AR up-regulates after chronic agonist exposure through transcriptional induction of multiple cAMP response elements in the receptor gene secondary to ligand-induced activation of the cAMP signaling pathway (12). Agonist-induced up-regulation...
has been extensively investigated in the case of the D2$_2$R, either as endogenous receptors in tumor cell lines or as recombinant receptors in various host cells (18–21). These studies have revealed a time- and concentration-dependent induction of surface receptors by 30–300% over 4–20 h by 10$^{-2}$–10$^{-6}$ M dopamine in different cells (17–19). The effect of cycloheximide on up-regulation of D2$_2$R is controversial, with some (18) but not all (19–21) studies reporting blockade of up-regulation by the protein synthesis inhibitor, cycloheximide. The kinetics of hSSTR1 up-regulation that we found were comparable to those of the D2LR. Thus hSSTR1 was up-regulated in a time-, temperature-, and dose-dependent manner to saturable levels. Up-regulation did not occur in membrane preparations, required the intact cell, and produced functional G protein-coupled surface receptors. Furthermore, up-regulation was unaffected by cycloheximide suggesting that it is not due to new receptor synthesis but likely represents receptors from a pre-existing pool. This is in agreement with earlier findings that up-regulation of endogenous SSTRs in GH4C1 cells (which express predominantly the SSTR1 subtype) are also insensitive to cycloheximide (22). Overall then, these results suggest that up-regulation of most GPCRs is dependent on transcriptional and posttranscriptional induction of new receptor synthesis, the exception being SSTR1 and probably the D2$_2$R.

Four of the five SSTR isotypes, SSTR2, -3, -4, -5, are readily internalized by ligand binding (23, 24, 27, 28). Internalization of SSTR2, -3, -5 has been shown to be dependent on residues in the C-tail (24, 27, 28), and in the case of hSSTR5 both negative and positive endocytic signals have been identified (24). Like the other SSTRs, the C-tail of hSSTR1 is rich in putative serine and threonine phosphorylation sites, and additionally features three tyrosine residues that could act as potential endocytic signals (Fig. 1). Nonetheless, this receptor was incapable of ligand-induced internalization. We found that the C-tail of hSSTR1 contains negative internalization signals, since substitution of the C-tail of hSSTR5 with that of hSSTR1 blocked internalization of the chimeric receptor. The inability of hSSTR1 to internalize, however, cannot be attributed solely to

![Fig. 8. Up-regulation of mutant and hSSTR1-hSSTR5 chimeric receptors.](image-url)

Whole cell binding analysis of mutant and chimeric receptors in stable CHO-K1 cells treated with SST-14 (10$^{-7}$ M) for 22 h. Up-regulation of hSSTR1 by chronic agonist treatment (A) is completely abolished by truncating the receptor C-tail (C). The hSSTR5 C-tail mutant (E) shows no up-regulation. Substitution of the hSSTR5 C-tail with that of hSSTR1 converts the chimeric receptor to one resembling wt hSSTR1 with 71% up-regulation of surface binding (D) (mean ± S.E. of three independent experiments).

![Fig. 9. Coupling of mutant and chimeric hSSTR1-hSSTR5 receptors to adenylyl cyclase.](image-url)

Dose-dependent inhibition by SST-14 of forskolin-stimulated cAMP in CHO-K1 cells stably expressing mutant and chimeric hSSTR1-hSSTR5 receptors (mean ± S.E. of three independent experiments in triplicate).
negative signals in the C-tail since deletion of the C-tail did not activate internalization suggesting that additional positive signals in the C-tail or on residues located in other intracellular domains are required. Our finding that SSTR1 is refractory to agonist-promoted endocytosis raises the question of whether the increased surface binding over time is simply a reflection of receptor aggregates, such as dimers with altered binding, or membrane accumulation of ligand-stabilized SSTRs that would otherwise have been degraded. Several other receptors that up-regulate, e.g., β2AR, D2LR are also resistant to internalization (21, 29–31), whereas others such as the GnRH receptor (15), 5HT2 receptor (32), and SSTR2 and -4 display both endocytosis and up-regulation (23, 27) suggesting that lack of internalization is not an absolute requirement for up-regulation. Likewise, up-regulation is not an automatic consequence of poor internalization as indicated by the Δ C-tail hSSTR1 mutant in the present study which displayed neither internalization nor up-regulation. We have recently reported that hSSTR1 and hSSTR5 associate as dimers both as homodimers or heterodimers and that dimerization alters the functional properties of the receptor such as ligand binding affinity and agonist regulation (33). Our finding, however, that up-regulation is temperature-dependent and does not occur when membranes are incubated directly with agonist rules out surface aggregation and points toward an active process of receptor recruitment to the plasma membrane. This was directly demonstrated by confocal fluorescence immunocytochemistry that showed a progressive time-dependent increase in surface SSTR labeling associated with a parallel depletion of intracellular SSTR immunofluorescent vesicles suggesting translocation from the cytoplasm to the plasma membrane.

Up-regulation of hSSTR1 was not dependent on receptor signaling as evident from the dissociated effects of the C-tail deletion mutants and chimeric receptors on up-regulation and coupling to adenylyl cyclase. For instance, the Δ C-tail hSSTR1 mutant showed complete loss of up-regulation in the face of only a small decrease in adenylyl cyclase coupling efficiency. Up-regulation was only partially inhibited by pertussis toxin implying that coupling to a second messenger system via pertussis toxin-sensitive G proteins such as G1 or Gq is not required. The involvement of non-G protein-linked pathways such as the Na+/H+ antiporter to which hSSTR1 is coupled, however, cannot be excluded (8). Dissociation of up-regulation from signaling has also been noted in the case of the D2L receptor that has been shown to up-regulate as efficiently with antagonists as with agonists suggesting that receptor occupancy irrespective of signaling capability is the critical determinant (20). This means that as in the case of the internalization of many GPCRs which can be dissociated from receptor signaling, up-regulation is an intrinsic property of some receptors such as hSSTR1, being triggered by a specific ligand-induced conformational change. The molecular signals that specify hSSTR1 up-regulation are located in the C-tail since deletion of this segment abrogated the up-regulation response. Even more compelling evidence that the C-tail of hSSTR1 harbors up-regulating sequences came from the chimeric receptor studies in which the C-tail of hSSTR1 conferred the property of up-regulation to hSSTR5, a receptor that normally displays agonist-dependent internalization. The nature of the molecular signals in the C-tail of hSSTR1 that mediate up-regulation remains to be determined. As in the case of internalization, phosphorylation of C-tail residues is likely to be important, given our finding that okadaic acid, an inhibitor of serine, threonine phosphatase, completely abolished up-regulation, suggesting that receptor dephosphorylation is a requisite step for up-regulation. But where is the cytoplasmic receptor pool that interacts with the receptor C-tail and what are the intervening steps? Since the receptor is not internalized, it is likely to be in a nonendosomal compartment, probably in post-Golgi transport vesicles for targeting the receptor to the plasma membrane. This is consistent with our immunocytochemical studies that showed that hSSTR1 is distributed in morphologically distinct cytoplasmic vesicles compared with the endosomal localization of hSSTR5. Recent work with the β-adrenergic receptor has proposed internalization as an obligatory requirement for activation of the mitogenic signaling complex (34). This model assigns a pivotal role to β-arrestin that binds to the ligand-activated, phosphorylated receptor and triggers the assembly of clathrin and the cytoplasmic tyrosine kinase c-src on β-arrestin molecules to initiate internalization of the receptor complex as a necessary step for effecting MAP kinase activation. The finding that SSTR1 can activate MAP kinase (35) without being internalized suggests that there may be alternative non-arrestin-dependent pathways for coupling the receptor to the MAP kinase signaling cascade.

In summary, these results show that hSSTR1 can desensitize rapidly in response to agonist but lacks the ability to be internalized, and thus displays only part of the acute agonist-dependent regulatory response. Continued agonist exposure induces a time- and concentration-dependent up-regulation of functional surface receptors. Up-regulation occurs by a temperature-dependent active process of ligand-induced receptor recruitment from a pre-existing cytoplasmic pool. It does not require new protein synthesis or signal transduction, is sensitive to dephosphorylation events, and is critically dependent on molecular signals in the receptor C-tail. Further studies are required to map the specific regulatory motifs in the C-tail of hSSTR1 and to identify the mediators distal to the C-tail which direct receptor trafficking from the cytoplasm to the plasma membrane.

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