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Agonist-induced up-regulation of human somatostatin receptor type 1 is regulated by β-arrestin-1 and requires an essential serine residue in the receptor C-tail

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Abstract

We have previously shown that the human somatostatin receptor type 1 (hSSTR1) does not undergo agonist-induced internalization, but is instead up-regulated at the membrane upon prolonged somatostatin (SST) exposure. The deletion of the carboxyterminal C-tail of the receptor completely abolishes up-regulation. To identify molecular signals that mediate hSSTR1 up-regulation, we created mutant receptors with progressive C-tail deletions. Up-regulation was found to be absent in mutants lacking residues Lys^{359}-Ser^{360}-Arg^{361}. Moreover, point mutation of Ser^{360} to Ala completely abolished up-regulation. The coexpression of wild type hSSTR1 with V53D, a dominant negative mutant of β-arrestin-1, completely blocked hSSTR1 up-regulation. Further analysis demonstrated that calcium-calmodulin (CaM) dependent kinases were essential for the SST-induced up-regulation response. Like wild type receptors, all mutants failed to internalize after agonist exposure and were able to inhibit forskolin-stimulated cAMP accumulation. Taking these data together, we suggest that SST-induced hSSTR1 up-regulation is critically dependent upon a specific Lys-Ser-Arg sequence in the C-tail of the receptor, with Ser^{360} being essential. Up-regulation also requires the participation of CaM protein kinases and interactions with β-arrestins. In contrast, coupling to adenyl cyclase (AC) and internalization occur independently of molecular signals in the receptor's C-tail.

Keywords: hSSTR1; Somatostatin; Up-regulation; Sequence motif; β-Arrestin; Kinase

1. Introduction

The two physiologically active forms of somatostatin, SST-14 and SST-28, mediate their actions by interacting with a family of five somatostatin receptors (SSTR1–5) of family A of the superfamily of seven transmembrane domain G protein-coupled receptors (GPCRs) [1–4]. In the case of human SSTRs, four of the isoforms (SSTR1–4) display weak selectivity for binding to SST-14, whereas hSSTR5 shows a preference for SST-28 [1]. Recently, the complexity of the somatostatinergic system has been increased by the discovery of cortistatin, a neuropeptide that displays strong structural similarity to somatostatin (SST) and binds and activates SSTRs [5]. SSTRs have a complex, overlapping pattern of expression (reviewed in [2] and [4]). SSTR1 is expressed in normal tissues such as the pituitary, pancreas, adrenal glands and retina, as well as in many tumor cell lines [2,4–8]. Endogenous SSTRs interact with different types of G proteins, with the adenyl cyclase (AC)-cAMP-protein kinase A (PKA) being one of the most widely studied intracellular effector pathways. SSTR1 triggers the inhibition of AC through interactions with pertussis toxin-sensitive G_i/G_o proteins in Chinese hamster ovary (CHO-K1) cells [9,10]. In addition, SSTR1 is involved in the inhibition of voltage-dependent Ca^{2+}

Abbreviations: AC, adenylyl cyclase; CaM, calcium calmodulin; CHO-KI, Chinese hamster ovary cell line; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; GRK, GPCR kinase; hSSTR, human somatostatin receptor; MAPK, mitogen activated protein kinase; PKA, protein kinase A; PTX, pertussis toxin; PLC, phospholipase C; PCR, polymerase chain reaction; SST, somatostatin; SSTR, somatostatin receptor

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channels in CH4C1 rat pituitary tumor cells [11], the activation of phospholipase C (PLC) in CHO-K1 and COS cells [11], the activation of Na^+/H^+ ion exchangers in mouse fibroblasts [12] and the activation of mitogen activated protein kinase (MAPK) in CHO cells [13].

SSTRs, like most GPCRs, regulate their responsiveness upon agonist exposure [1,2,4,14]. The exposure of most GPCRs to their agonists results in a rapid attenuation of receptor responsiveness, a phenomenon called desensitization. This process usually results from a combination of different mechanisms, e.g., receptor phosphorylation and uncoupling from G proteins, internalization of cell surface receptors, often by clathrin-mediated endocytosis, and receptor downregulation due to reduced receptor synthesis combined with increased degradation of pre-existing receptors [2,15]. Phosphorylation of Ser and/or Thr residues by different protein kinases [2,15,16] and binding to a family of scaffolding proteins called β-arrestins [17,18] are key steps in receptor desensitization and internalization processes. In the last few years, there has been increasing evidence that β-arrestins also play a role in recruiting additional signaling molecules to activated receptors [17,18]. Recently, Tulipano et al. [19] demonstrated a role for β-arrestin in SSTR1 trafficking. Furthermore, several studies have shown agonist-induced desensitization and internalization of native SSTRs (reviewed in [4]). SSTR2, 3, 4 and 5 undergo rapid internalization upon agonist treatment (reviewed in [2] and [4]) while SSTR1 has been reported to show no or poor internalization (reviewed in [2,20–22]).

The up-regulation of SSTRs by long-term agonist treatment has been described in pituitary and islet tumor cells [23,24]. Furthermore, it has also been reported for other GPCRs, e.g., β3-adrenergic receptor [25], 5HT2A [26], GnRH receptor [27], angiotensin II receptor [28], and the long form of dopamine 2 receptor [29]. SSTR2 subtype mRNA is increased in rat hypothalamic arcuate nucleus 3 h after octreotride, a SST analog, injection [30]. However, unlike receptor desensitization and internalization, the underlying molecular mechanisms implicated in up-regulation are poorly understood [21]. We have previously reported that hSSTR1 is up-regulated in stably transfected CHO-K1 cells upon agonist exposure, in a time-, temperature- and dose-dependent fashion [21]. The truncation of the cytoplasmic carboxyterminal tail (C-tail) of the receptor completely abolishes this effect of SST, indicating that up-regulation is critically dependent on molecular signals in the receptor C-tail [21]. Therefore, the specific objective of the present study is to establish the specific motif in the hSSTR1 C-tail that determines agonist-induced up-regulation of the receptor, as well as which secondary messenger pathways are involved. The results demonstrate that the homologous up-regulation of hSSTR1 is regulated by β-arrestin-1 and calcium-calmodulin (CaM) dependent kinases, and requires an essential Ser residue in the C-tail of the receptor.

2. Materials and methods

2.1. Materials

SST-14, SST-28 and Leu^8, $D\text{-}Trp^{22}$, Tyr^{25} SST-28 (LTT-SST-28) were obtained from Bachem (Marina del Rey, CA). Ham's F-12 medium, fetal bovine serum (FBS), neomycin (G418) and hygromycin B, were from Gibco BRL. Forskolin, brefeldin A, phenylarsine oxide, cholera toxin, *N*-(2-[*p*-Bromocinnamylamino]ethyl)-5-isoquinoline sulfonamide (H-89), 2-[2-amino-3-methoxyphenyl]-4*H*-1-benzopyran-4-one (PD098,059), dextran sulfate, phorbol 12-myristate 13-acetate (PMA), heparin, bisindoylmaleimide (GF109203X) and *N*-[2-(*N*-(4-chlorocinnamyl)-*N*-methyl aminomethyl) phenyl]-*N*-[2-hydroxethyl]-4-methoxy benzene-sulfonamide (KN-93) were purchased from SIGMA (Sigma-Aldrich Canada Ltd.). Cyclic AMP radioimmunoassay kits were obtained from Diagnostic Products Corp. (Los Angeles, CA). Non-peptide subtype selective agonists for SSTR1 and SSTR2 were kindly supplied by Dr. S.P. Roher and Dr. J.M. Schaeffer. cDNAs for wild type (wt) β-arrestin-1 and its corresponding dominant negative mutant, V53D, were helpfully donated by Dr. J.A. Holt and Dr. M. Caron. All other reagents were purchased from various suppliers as indicated below.

2.2. Construction of wild type, mutant and chimeric receptors and β-arrestin cDNAs

Wt hSSTR1 and hSSTR5 cDNAs encoding the complete receptor sequences were generated by PCR amplification using previous cassette construct DNAs as templates [21] and subcloned into *Hin*dIII/*Eco*R1 multiple cloning sites of pcDNA3.1(+) (Invitrogen, Carlsbad, CA) expression vectors. A series of progressive deletion mutants of the hSSTR1 C-tail were created by PCR using oligonucleotide primers that contained appropriately placed stop codons. The stop codons were created at positions 337, 347, 354, 358, 361, 371 and 381 of the hSSTR1 C-tail sequence and mutant receptors were named according to the stop codon position (Fig. 1). The chimeric receptors R5CR1 (hSSTR5 receptor with the fragments 328–361 of the hSSTR1 C-tail), and D328–347 (hSSTR1 receptor lacking the fragment 328–347 in the C-tail), and point mutants C339A and S360A were created by the PCR overlap extension technique [21]. PCR techniques similar to those used for the wild type receptors were used to clone the wt human β-arrestin-1 and dominant negative β-arrestin-1 mutant V53D [31,32] into the *Hin*dIII/*Xho*I cloning sites of pcDNA3.1(+)/Hygro expression vectors. All primers were designed to avoid changes in the reading frame. The primers used for the construction of different mutant and wild type receptors are shown in Table 1.

2.3. Polymerase chain reaction (PCR)

PCR reactions were carried out with 300 ng of SSTR or β-arrestin cDNA template in 100 μl containing 20 mM

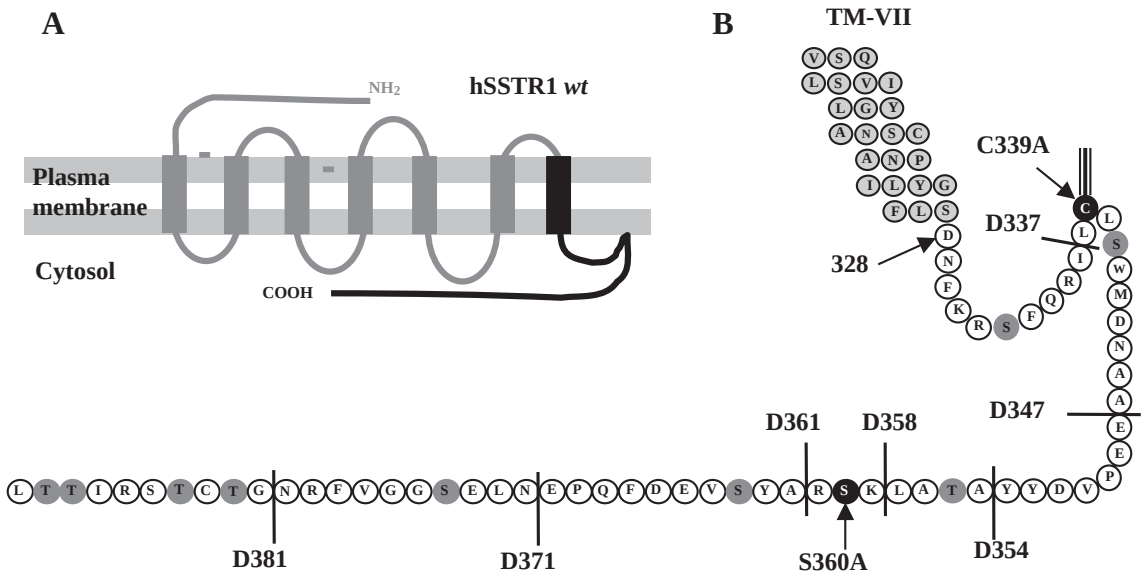

Fig. 1. Schematic depiction of the topology of hSSTR1. Panel A depicts the putative membrane topology of hSSTR1 (total of 391 amino acid residues). The seventh transmembrane domain (TM-VII) and C-tail are shaded in black. Panel B shows the amplified detailed amino acid sequence of the C-tail to illustrate the structure of the different mutant receptors that were constructed. Stop codons were introduced at residues 337, 347, 354, 358, 361, 371 and 381 to create truncated receptors with variable length C-tail (D337, D347, D354, D358, D361, D371 and D381). The two residues mutated to Ala, Cys^{339}, a putative palmitoylation site, and Ser^{360}, the essential residue for up-regulation (see text), are highlighted in black (C339A and S360A). Putative phosphorylation Ser and Thr residues in the C-tail are shown as shaded gray circles. TM-VII residues are shaded in light gray. Arrows indicate the residue number.

Tris–HCl, pH 8.5, 50 mM KCl, 200 μM dNTPs, 1.5 mM $MgCl_2$, 5% DMSO, 15 pmol of each primer, and 2.5 units of Pfu polymerase (Stratagene). PCR products were separated by agarose gel electrophoresis and the amplified bands were purified. For the chimeric receptors, fragments were fused in a third PCR reaction to generate full-length chimeric receptors using junction primer pairs (Table 1). All 5′-flanking primers contained *Hin*dIII endonuclease restriction sites, Kozak consensus sequences, and initiation codons. All 3′-flanking primers were comprised of a stop codon followed by an *Eco*RI or *Xho*I restriction site. The PCR products were digested with the appropriate restriction enzymes and purified fragments were subcloned into the multiple restriction sites of different expression vectors.

Table 1
Oligonucleotide primers used in the creation of hSSTR1 C-tail deletion mutants, chimeric and wild type receptors

Oligo	Sequence
hSSTR1 forward	5′ ATTCAT AAGCTT GCCGCCACC ATGTTCCCCAATGGCA 3′
hSSTR1 reverse	5′ TAGTAGATGAATTCATCAGAGCGTCGTGAT 3′
D337 reverse	5′ ATATCGAATTCACTAAGATGCGTTGGAAAG 3′
D347 reverse	5′ CGATCGAATTCACTACGCGGCGTTGTCCA 3′
D361 reverse	5′ CGATCGAATTCACTAACGGCTCTTGAGC 3′
D371 reverse	5′ CGATCGAATTCACTACTCAGGTTGGAAG 3′
D381 reverse	5′ CGATCGAATTCACTAATTACGGAAGACGC 3′
D328–347 junction forward	5′ TATGGCTTTCTCTCAGAGGAGCCGGTTGA 3′
D328–347 junction reverse	5′ TCAACCGGCTCCTCTGAGAGAAAGCCATA 3′
D354 reverse	5′CGATCGAATTCACTAGTAATAGTCAACCGGCT3′
D358 reverse	5′ CGATCGAATTCACTAGAGCGCGGTGGCGTA 3′
hSSTR5 forward	5′ ATTCAT AAGCTT GCCGCCACC ATGGAGCCCCTG TT 3′
R5-R1 junction forward	5′ TCTGACAACTTCAAGCGCTCTTT 3′
R5-R1 junction reverse	5′ AAAGAGCGCTTGAAGTTGTCAGA 3′
C339A junction forward	5′ ACG CAT CCT AGC CCT CAG CTG GA 3′
C339A junction reverse	5′ TCCAGCTGAGGGCTAGGATGCGT 3′
S360A junction forward	5′ TCAAGGCCCGTGCCTACAGT 3′
S360A junction reverse	5′ ACTGTAGGCACGGGCCTTGA 3′
β-Arrestins forward	5′ ATTCAT AAGCTT GCCGCCACC ATGGGCGACAAAGGGA 3′
β-Arrestins reverse	5′ TAGTAGATCTCGAGACTATCTGTTGTTGAGCT 3′

Restriction sites used for cloning into expression vectors are underlined.

Plasmid vectors containing the different inserts were isolated from transformed bacteria (DH5α™ Competent Cells, Gibco BRL) using the EndoFree Plasmid Purification Kit (Qiagen Inc., Canada). The structure of all receptor constructs was confirmed by sequence analysis (University Core DNA Service, University of Calgary, Calgary, Alberta, Canada). CHO-K1 cells were transfected with 5 μg cDNAs, cloned into suitable expression vectors using the Superfect Transfection Reagent method (Qiagen), and stable G418-resistant and Hygromycin-resistant (for cotransfections with β-arrestins) cells were propagated for the study.

2.4. Binding studies

Transfected CHO-K1 cells were cultured in D75 flasks to ~90% confluency in Ham's F-12 medium containing 10% fetal calf serum and 750 μg/ml G418. Cells were harvested and homogenized and membranes were prepared by centrifugation as previously described [21]. Binding studies were carried out for 35 min at 37 °C with 40–50 μg of membrane protein and ^{125}I-LTT-SST-28 radioligand in 50 mM Hepes, pH 7.5, 2 mM $CaCl_2$, 5 mM $MgCl_2$, 0.2% BSA, 0.02% phenylmethylsulfonil fluoride, and 0.02% bacitracin (binding buffer). Saturation binding experiments were performed with membranes using increasing concentrations of ^{125}I-LTT-SST-28 under equilibrium binding conditions [21,33]. Radioactivity associated with membrane pellets was quantified in an LKB gamma counter (LKB-Wallach, Turco, Finland). Data analysis and calculation of kinetic constants (K_d and B_{max}) were performed by nonlinear regression analysis using Prism 3.0 (Graph Pad Software, San Diego, CA). Values were expressed as mean±S.E. of three independent experiments.

2.5. Up-regulation experiments

Transfected cells were cultured in 6-well plates to ~90% confluency in F-12 medium with serum and G418. At the onset of the experiment, medium was removed and replaced with F-12 medium without serum and without antibiotics, with or without 100 nM SST-14. Cells were subsequently incubated for 22 h at 37 °C. For the intracellular signaling inhibition experiments, cultures were treated with 100 nM SST-14 alone or in combination with the specific inhibitory substances (see Table 3 for details). After acid wash (Hank's-buffered saline acidified to pH 5.0 with 20 mM sodium acetate) to remove surface-bound SST, whole cell binding assays were performed to determine total and nonspecific binding [21]. Residual surface binding was calculated as the difference between the control and experimental groups. Radioactive fractions were counted in a LKB gamma counter. Each experiment was performed at least 4 times in triplicate.

2.6. Internalization experiments

Transfected CHO-K1 cells were cultured in 6-well plates to ~90% confluency in F-12 medium with serum and G418. Cells were equilibrated overnight at 4 °C with ^{125}I-LTT-SST-28 with or without 100 nM SST-14. After washing, cells were warmed to 37 °C for 60 min to initiate internalization. At the end of the incubation period, surface bound radioligand was removed by treatment with 1 ml of acid wash for 8 min at 37 °C. Internalized radioligand was measured as the acid-resistant count in 0.1 N NaOH extracts of acid-washed cells [21,33]. Radioactive fractions were counted in an LKB gamma counter. Each experiment was performed at least 3 times in triplicate.

2.7. Coupling to adenylyl cyclase

Transfected cells were incubated in 6-well plates to ~90% confluency. Receptor coupling to AC was tested by incubating cells for 30 min with 1 μM forskolin and 0.5 mM 3-isobutyl-1-methylxanthine (SIGMA), with or without 1 μM SST-14, at 37 °C as described previously [21]. Cells were scraped in 0.1 N HCl, sonicated, and centrifuged at 3000 rpm for 20 min. Supernatants were kept at −20 °C until cAMP quantification by radioimmunoasay.

2.8. Statistical analysis

Data are expressed as mean±S.E. obtained from at least three independent experiments performed in triplicate. Statistical analysis was carried out using a one-way ANOVA, followed by a Student's unpaired *t* test, comparing with the appropriate control (wt hSSTR1 or SST-treated cells). *P* values <0.05 were considered statistically significant.

3. Results

3.1. Binding characteristics of C-tail deletions and chimeric receptors

All receptors were correctly targeted to the plasma membrane and were able to bind the ligand, as determined by binding analysis (Table 2). Saturation binding analysis in transfected CHO-K1 cells revealed a comparable level of expression (Bmax) for all receptors, including wt hSSTR1 and hSSTR5, and mutants (~190 fmol/mg protein). All mutant and chimeric receptors presented a comparable binding affinity (K_d) to that of wt hSSTR1 and hSSTR5 (Table 2). Moreover, chimeric receptor R5CR1 showed the same binding affinity and level of expression as wt hSSTR5. These results, in agreement with our previous studies [21,32], demonstrate that the hSSTR1 and hSSTR5 C-tails, like that of many GPCRs [34], do not participate in receptor targeting or binding conformation. Likewise, the cotransfec-

Table 2
Comparison of binding, up-regulation, internalization, and adenylyl cyclase coupling of wild type, mutant and chimeric receptors transfected in CHO-K1 cells

Receptor	K_d (nM)	B_{max} (fmol/mg protein)	Up-regulation Percentage (%) of binding increase at 22 h	Internalization Percentage (%) at 60 min	Adenylyl cyclase coupling Percentage (%) inhibition of FSK-stimulated cAMP
hSSTR1	0.53±0.17	195.7±13.9	98.3±17.3	2.49±1.45	45.31±2.1
D337	0.72±0.05	182.6±9.25	0***	2.95±1.32	39.30±7.7
D347	0.44±0.10	188.1±7.89	0***	2.85±1.50	32.80±7.2
D354	0.63±0.09	176.1±5.70	0***	1.36±0.80	39.80±5.2
D358	0.56±0.10	208.7±19.0	0***	3.47±2.35	44.91±4.8
D361	1.25±0.58	162.1±11.8	63.7±7.12	2.42±1.15	28.26±10
D371	0.91±0.25	185.5±2.04	77.4±13.86	1.24±1.24	51.23±11.9
D381	0.45±0.04	192.3±30.7	69.1±10.24	4.26±2.22	41.58±12.9
D328-347	0.63±0.14	166.4±7.80	35.5±10.3**	1.14±0.89	40.05±7.4
R5CR1	0.95±0.46	200.5±7.82	122.0±18.14	1.60±1.08	31.6±6.2
hSSTR5	0.43±0.13	198.2±12.8	0***	49.86±1.54***	45.2±8.4
C339A	0.51±0.02	196.3±6.26	32.1±9.05*	2.66±1.55	52.22±3.6
S360A	0.45±0.03	204.7±8.04	0	3.33±2.11	44.62±3.6
hSSTR1+β-arrestin-1	0.74±0.14	201.5±42.0	76.9±9.01	15.7±1.96***	41.97±4.5
hSSTR1+V53D	0.74±0.26	185.1±12.5	0***	4.03±2.16	56.01±3.7

Data are the mean±S.E. of at least three independent experiments.
* $P<0.05$ vs. hSSTR1.
** $P<0.01$ vs. hSSTR1.
*** $P<0.001$ vs. hSSTR1.

tion of wt hSSTR1 with wt β-arrestin-1 or mutant V53D did not induce any significant changes in B_{max} or K_d (Table 2).

3.2. Determination of the molecular signals in the hSSTR1 cytoplasmic C-tail required for agonist-induced up-regulation

Previously, we have shown that ligand-induced up-regulation of hSSTR1 is critically dependent on molecular signals in the receptor's C-tail [21]. Therefore, the specific goal of the present study was to determine the amino acid motif in the receptor C-tail implicated in the process of up-regulation. Accordingly, we constructed a series of progressive truncation mutants of the C-tail (D337, D347, D361, D371 and D381, Fig. 1) and used them for stable transfections into CHO-K1 cells. The results of whole cell binding analysis of wt hSSTR1 and specific C-tail deletion mutants treated with SST-14 for 22 h are described in Table 2. In concordance with our previous report [21], wt hSSTR1 undergoes up-regulation (98.3±17.3% over untreated control cells) in surface binding after 22 h of exposure to SST-14 (100 nM). The deletion of the C-tail at residues 337 and 347 completely abolished this up-regulation, whereas mutants D361, D371 and D381 displayed an up-regulation similar to that of wt hSSTR1 (Table 2). These results strongly suggest that the proximal half of the hSSTR1 C-tail, and particularly the fragment ^{348}EEPVDYYA-TALKSR^{361}, contains molecular signals crucial for up-regulation (Fig. 1).

We then further narrowed down the specific motif within the 347–361 sequence that controlled hSSTR1 up-regulation. With this aim, we created two new deletion mutants, at positions 354 and 358 (D354 and D358, Fig. 1). Likewise, to confirm the importance of the proximal part of the C-tail of hSSTR1 in up-regulation, we created a chimeric mutant, swapping residues 328–361 of the hSSTR1 C-tail with the hSSTR5 C-tail (mutant R5CR1). Furthermore, to determine if the fragment 348–361 of the hSSTR1 C-tail is sufficient to induce the up-regulation of the receptor, we also created a new chimeric receptor, lacking the fragment 328–347 of the C-tail but leaving the rest of the C-tail intact (mutant D328–347, Fig. 1). The truncation of the hSSTR1 C-tail at residues 354 or 358 completely abolished SST-induced up-regulation of the receptor (Table 2). With these results, we narrowed down the motif essential for hSSTR1 up-regulation to ^{359}Lys-Ser-Arg^{361} in the C-tail of the receptor (Fig. 1). Ser^{360}, a putative phosphorylation site, was expected to be the essential amino acid residue in this sequence. The substitution of the hSSTR5 C-tail with the hSSTR1 C-tail fragment 328–361 converted the chimeric receptor to one resembling wt hSSTR1, showing a similar capacity for up-regulation at the cell surface after long term SST treatment. In this set of experiments we used hSSTR5 as a negative control, since this SSTR subtype is not up-regulated (Table 2). Similar results for hSSTR5 and R5CR1 were obtained using SST-28 (data not shown). These results demonstrate that the molecular signal in the hSSTR1 C-tail required for receptor up-regulation is sufficient to induce a gain-of-function in a different receptor subtype. Surprisingly, the chimeric receptor D328–347 showed a reduced, but not completely abolished, up-regulation after SST challenge despite the fact that this receptor contains the crucial 348–361 fragment (Table 2). Therefore, the segment of the C-tail proximal to the seventh transmembrane domain (TM-VII) is also important, but not essential, in hSSTR1 up-regulation and probably helps create the most optimal secondary

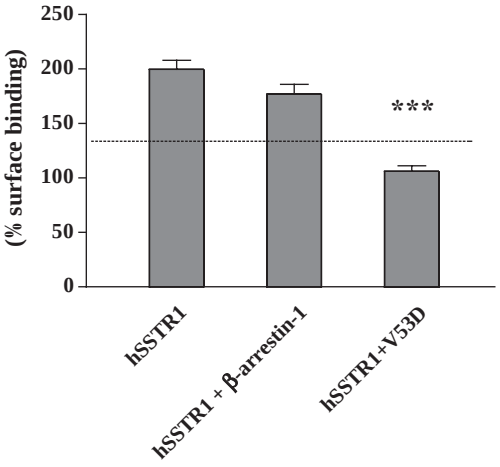

Fig. 2. Up-regulation of surface binding on CHO-K1 cells stably cotransfected with hSSTR1 and wt β-arrestin-1 or V53D β-arrestin-1 dominant negative mutant. CHO-K1 cells monotransfected with hSSTR1 and cotransfected with β-arrestin-1 or V53D were incubated with 100 nM SST-14 for 22 h at 37 °C. Whole cell binding was subsequently quantified as described in Materials and methods. The dotted line (100%) represents binding in the control cells from each cell line. N=9. ***P<0.001 vs. hSSTR1 monotransfected cell line.

structure of the C-tail. In this regard, this fragment contains the residue Cys^{339}, a palmitoylated cysteine residue that acts as an anchor to the plasma membrane.

To further confirm the importance of residue Ser^{360}, as a putative phosphorylation site, and Cys^{339}, as an anchor site, in SST-induced hSSTR1 up-regulation, we created two point mutant receptors by creating single point mutations whereby these two residues were replaced by Ala (mutants C339A and S360A, Fig. 1). As described in Table 2, receptor C339A showed a reduced, but not completely eliminated, up-regulation following SST treatment, showing a similar behavior to that of mutant D328–247. Furthermore, the mutation of Ser^{360} to Ala completely abolished SST-induced hSSTR1 up-regulation, confirming the fact that this amino acid residue is essential for SSTR1 up-regulation and strongly suggesting its participation in phosphorylation events (Table 2).

3.3. Determination of intracellular pathways implicated in hSSTR1 up-regulation

To determine the role played by β-arrestins in SST-induced hSSTR1 up-regulation, we performed cotransfections of hSSTR1 with wt β-arrestin-1 or mutant V53D. After 22 h treatment with SST, membrane binding was quantified (Table 2, Fig. 2). The coexpression of hSSTR1 with wt β-arrestin-1 only induced a slight, but not significant, reduction in hSSTR1 up-regulation following SST treatment, presumably due to an increase in receptor internalization (see below). On the other hand, the coexpression of hSSTR1 with the negative mutant V53D completely abolished the increase in SST-induced membrane binding, demonstrating the essential role played by β-arrestin-1 in hSSTR1 up-regulation (Table 2, Fig. 2). The overexpression of β-arrestin-1 in cotransfected cells was confirmed by immunocytochemistry (data not shown).

Subsequently, we characterized other signaling pathways implicated in up-regulation by challenging CHO-K1 cells transfected with hSSTR1 with 100 nM SST-14 for 22 h in the presence or absence of some inhibitors and stimulators of several key intracellular molecules (Table 3). None of the compounds, when tested alone, induced significant changes in the percentage of membrane binding. The concentrations used were chosen as the maximal dose that did not induce significant increases in cell death or cause any visible changes in cell morphology, as visualized under the microscope, after a 22 h treatment.

Table 3
Effects of different inhibitors and stimulators of intracellular signaling pathways on SST-induced hSSTR1 up-regulation in CHO-K1 cells after 22 h treatment

Compound	Action	Concentration used (M)	Up-regulation (% at 22 h)
Somatostatin-14		10^{-7}	100.8±5.6
Forskolin	Activates adenylyl cyclase	2×10^{-7}	84.1±7.9
H-89	Inhibits PKA	10^{-6}	92.3±15.4
PMA	Activates PKC	2×10^{-6}	113.1±11.2
GF109203X	Inhibits PKC	10^{-5}	107.3±7.6
Cholera toxin	Activates α-subunit of G proteins	10^{-7}	68.0±9.46*
Brefeldin A	Inhibits trafficking from Golgi to the cell surface	7×10^{-7}	41.0±13.9**
Sucrose	Disrupts endocytosis via clathrin coated pits	0.1	94.8±9.6
PAO	Inhibits internalization of membrane receptors	10^{-7}	87.2±7.5
PD098,059	Inhibits MAPKK	10^{-5}	110.0±10.0
Dextran sulfate	Inhibits GRKs	10^{-5}	80.4±5.1
Heparin	Inhibits GRKs	10^{-5}	41.3±10.0**
KN-93	Inhibits Ca^{2+}-Calmodulin kinases	10^{-5}	7.3±7.8***

Data are the mean±S.E. of at least three independent experiments.
None of the chemical compounds have a significant effect on surface binding.
N=6.
* P<0.05 vs. SST-14 alone.
** P<0.01 vs. SST-14 alone.
*** P<0.001 vs. SST-14 alone.

The effects of the different compounds upon hSSTR1 up-regulation are displayed in Table 3. Forskolin (activator of AC), H-89 (inhibitor of PKA), PMA and GF109203X (an activator and inhibitor, respectively, of PKC) had no effect on SST-induced hSSTR1 up-regulation, indicating that PKA and PKC do not participate in SSTR1 up-regulation. Treatment of hSSTR1 cells with cholera toxin reduced up-regulation by 30%, suggesting the participation of G proteins in hSSTR1 up-regulation (Table 3). Brefeldin A, a disruptor that inhibits trafficking from the Golgi apparatus to the cell surface, inhibited up-regulation by ~60% (Table 3), suggesting the Golgi as the source of the up-regulated receptors. Moreover, the endocytic machinery that normally regulates the internalization of GPCRs seems to not be necessary in hSSTR1 up-regulation, since sucrose and phenylarsine oxide, two inhibitors of internalization processes, did not have any effect on hSSTR1 up-regulation (Table 3). In regard to other protein kinases that might be implicated in the up-regulation process, PD098,059, an inhibitor of MAPKK, did not induce any significant changes, suggesting that these MAPKs are not involved in the process of up-regulation. On the other hand, dextran sulfate and heparin, inhibitors of GPCR kinases (GRKs), were able to reduce SST-induced hSSTR1 up-regulation, although this inhibition was only significant for heparin. These data suggest the involvement of GRKs in up-regulation, but, given the different effects of the two inhibitors, it might be a specific GRK-dependent process. Significantly, KN-93, an inhibitor of CaM kinases totally abolished receptor up-regulation after SST treatment (Table 3), strongly suggesting the participation of these protein kinases.

3.4. Specificity of the hSSTR1 up-regulation response

The ability to be regulated by ones own natural ligand is a common property of most GPCRs and is called homologous regulation. To determine the specificity of the hSSTR1 up-regulatory response, we performed a series of experiments challenging CHO-K1 cells transfected with hSSTR1 with cortistatin, a newly discovered neuropeptide displaying a strong structural similarity to SST [5], and with L-797591, a non-peptidic agonist specific for SSTR1 [35]. Long term (22 h) treatment with 100 nM cortistatin induced a similar degree of hSSTR1 up-regulation as 100 nM SST-14 did (Fig. 3). Surprisingly, a 1 μM concentration of the non-peptidic analog was only able to induce a slight, although significant, increase in membrane binding. However, despite its limited ability to induce up-regulation, this analog was able to inhibit forskolin-stimulated cAMP increases in CHO-K1 cells transfected with hSSTR1 to a similar degree to that observed with SST (data not shown). 1 μM of the non-peptidic analog (L-779976), a specific analog for SSTR2 used here as a negative control, did not elucidate any changes in membrane binding (Fig. 3). These data demonstrate that hSSTR1 up-regulation is a homologous phenomenon, and that specific conformational changes in the receptor might be necessary.

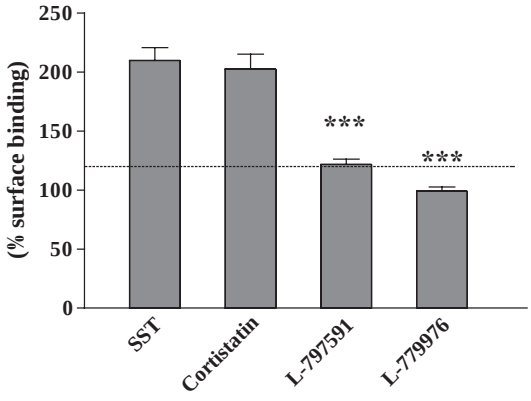

Fig. 3. Homologous up-regulation of surface hSSTR1 in stably transfected CHO-K1 cells. Cells expressing hSSTR1 were incubated for 22 h at 37 °C with serum-free F-12 medium (control, dotted line, 100%) alone or in the presence of 100 nM SST-14, 100 nM cortistatin, 1 μM L-797591 (nonpeptide selective agonist for SSTR1) or 1 μM L-779976 (nonpeptide selective agonist for SSTR2). After treatment, whole cell binding was performed as described in Materials and methods. N=3. ***P<0.001 vs. hSSTR1.

3.5. Internalization of wt hSSTR1, chimeric and mutant receptors

Table 2 depicts the internalization results of transfected CHO-K1 cells incubated for 60 min with ^{125}I-LTT-SST-28. As previously reported [21], hSSTR5 undergoes rapid internalization (49.86±1.54%) after a 60 min incubation at 37 °C, whereas hSSTR1, under comparable incubation conditions, shows no significant internalization. As anticipated from our previous work, swapping the C-tail of hSSTR5 with the 328–361 fragment of the hSSTR1 C-tail (mutant R5CR1) completely abolishes hSSTR5 internalization, converting the chimeric receptor to one resembling wt hSSTR1. All C-tail truncation mutants, the chimeric D328–347, and the point mutant receptors displayed comparable inabilities to undergo agonist-promoted endocytosis to that of hSSTR1. These results demonstrate that the lack of hSSTR1 internalization is not due to the presence of negative internalization signals in its C-tail, but to the lack of positive internalization signals. Not totally unexpected, co-transfection with wt β-arrestin-1 increases hSSTR1 internalization to ~15% (Table 2), probably due to the overexpression of the β-arrestin-1 protein. In contrast, the coexpression of SSTR1 with the V53D negative mutant did not enhance SSTR1 internalization.

3.6. Coupling of hSSTR1, chimeric and mutant receptors to adenylyl cyclase

In order to determine the influence of receptor signaling capability on the hSSTR1 up-regulation process, we determined the ability of wt, mutant and chimeric receptors

to inhibit forskolin-stimulated cAMP accumulation by SST-14 (Table 2). As previously reported, both hSSTR1 and hSSTR5 are functionally coupled to AC. Surprisingly, the mutant R5CR1 retains its ability to inhibit forskolin-stimulated cAMP, showing a slightly lower, but not significantly different, capacity for inhibition (~32%) as hSSTR5 (Table 2). As with the internalization process, none of the hSSTR1 C-tail mutations significantly modified the ability of the receptor to inhibit forskolin-stimulated cAMP accumulation. However, mutants D347 and D361 showed slightly lower, but not significant, inhibition capacities than that of the wt receptor. Likewise, the coexpression of hSSTR1 with wt β-arrestin-1 did not modify the inhibitory capacity of the receptor. These results strongly suggest that the functional coupling of hSSTR1 to AC is neither mediated by its C-tail nor through the participation of β-arrestin-1.

4. Discussion

4.1. Role of hSSTR1 C-tail in mediating receptor internalization

For most GPCRs, desensitization after agonist treatment is usually followed by endocytosis of the ligand–receptor complex which plays a key role in receptor downregulation, resensitization and signaling [4]. It has been demonstrated that the internalization of hSSTR2, 3 and 5 is dependent on residues in the receptors' C-tails [36,37], and, in the case of hSSTR5, both negative and positive endocytic signals have been identified [33]. In our hands, hSSTR1 is virtually unable to undergo internalization in stably transfected CHO-K1 cells. Moreover, R5CR1, a hSSTR5 mutant with the proximal half of the hSSTR1 C-tail, is also refractory to agonist-promoted endocytosis. Furthermore, none of the hSSTR1 deletion mutants are able to internalize upon agonist stimulation, which leads us to believe that the participation of other receptor domains are required for hSSTR1 internalization. Therefore, we hypothesize that the inability of hSSTR1 to undergo internalization is not due to the presence of negative signals, but rather due to the lack of positive internalization signals in the C-tail.

It is well known that β-arrestins play an essential role in the internalization of almost all GPCRs [15,31]. In our cell model, the cotransfection of hSSTR1 with wt β-arrestin-1 induced a slight, yet significant, increase in receptor internalization capacity. The cause for the differential internalization abilities of hSSTR1 and hSSTR5 is not known. However, it has been reported that the affinity of β_1-adrenergic receptor (β_1AR) for β-arrestins is lower than that of β_2AR, which explains β_1AR resistance to agonist-induced internalization [38,39]. Likewise, in our case, the overexpression of β-arrestin-1 might compensate, at least partially, for the low affinity of hSSTR1 for β-arrestins, thereby increasing the percentage of receptors internalized. However, in contrast to our observations, Tulipano et al. [19] presented data showing that hSSTR1 does not recruit β-arrestin-1. The reason for this discrepancy is not known and further studies are required. Furthermore, S360A and the other mutants which are not up-regulated interact differently from the wt receptor. β-arrestin-1 binding needs to be elucidated and further studies are in progress in this direction.

4.2. Role of hSSTR1 C-tail in coupling to G protein and adenylyl cyclase

The C-tail as well as the second and third intracellular loops of several GPCRs have been implicated in G protein interactions and receptor signaling ([14], reviewed in [3]). It is now well established that agonist binding to SSTR1 triggers the inhibition of AC throughout $G\alpha_{i3}$ and $G\alpha_{i1/2}$ in CHO-K1 cells expressing the human [40] and rat [41] isoforms of SSTR1, respectively. In our study, all mutant receptors showed a similar ability to inhibit forskolin-stimulated cAMP to that of wt hSSTR1. The results presented here, along with previous data [21], demonstrate that the hSSTR1 C-tail is not required for the inhibitory regulation of AC. These observations further suggest that hSSTR1 up-regulation and signaling are two independent processes. In contrast, it has been described that the progressive C-tail deletion mutants of hSSTR5 display a reduced efficiency for AC coupling [33]. In addition, the naturally occurring SSTR2B splice variant, which has a shorter C-tail than SSTR2A, is more efficiently coupled to AC [36].

4.3. Characterization of agonist-induced hSSTR1 up-regulation

We have previously shown that hSSTR1 is up-regulated after long term treatment with SST producing functional G protein-coupled surface receptors. Furthermore, truncation of the hSSTR1 cytoplasmic C-tail completely abolishes its up-regulation, indicating that the up-regulation process is critically dependent on specific molecular signals within the receptor's C-tail [21]. In order to map the specific regulatory motif, we created progressive deletion mutants. Given that all deletions performed before residue 361 completely abolished up-regulation, we found that ^{359}Lys-Ser-Arg^{361} is an essential motif in the regulation of agonist-induced hSSTR1 up-regulation. The fact that the chimeric receptor R5CR1 is able to undergo up-regulation like hSSTR1 demonstrates that this motif is enough to induce the up-regulation of a given receptor. It remains to be determined if this three-amino acid sequence is by itself a positive up-regulation motif, or part of a larger sequence. However, it is necessary to point out that mutant D328–347, which lacks the twenty proximal residues of the C-tail but includes the ^{359}Lys-Ser-Arg^{361} motif, displayed a reduced, but not complete, up-regulation response. hSSTR1 contains a

conserved Cys residue 12 amino acids downstream from the seventh transmembrane domain that is believed to be the site of a potential palmitoyl membrane anchor [1]. This cysteine anchor might give stability to the secondary and tertiary structures of the receptor, allowing a better interaction between the up-regulation motif and the putative intracellular protein(s) that triggers up-regulation. The fact that the point mutation of Cys^{339} induces a reduction in up-regulation supports this hypothesis. Agonist-induced phosphorylation of cytoplasmic residues, especially in the C-tail, has been reported for different SSTRs [2,20,42,43]. However, the phosphorylation of GPCRs at Ser and Thr, upon agonist stimulation, has been mainly associated with desensitization processes [15]. In this sense, rapid agonist-stimulated phosphorylation of rat SSTR1, mainly at Ser residues, has been reported to correlate with receptor desensitization, but not with internalization [20]. We have previously shown that okadaic acid, an inhibitor of Ser-Thr phosphatases, completely abolished hSSTR1 up-regulation [21]. In the present study, the mutation of Ser^{360} to Ala completely eliminated the up-regulation response, demonstrating that phosphorylation of the receptor is a necessary step to trigger SST-induced hSSTR1 up-regulation. Furthermore, based on previous studies using okadaic acid, a subsequent dephosphorylation process also seems to be needed. However, Ser^{360} could also be part of a previously described sequence for binding to PDZ domain proteins [44]. It has been shown that the rat SSTR2 C-tail interacts with the PDZ domain of a contacting-binding protein, establishing a physical link between a SSTR and constituents of the cytoskeleton protein. This physical link could be important for intracellular trafficking of the receptor [44]. Other unknown proteins might also interact with the ^{359}Lys-Ser-Arg^{361} motif in the hSSTR1 C-tail to control up-regulation. In this regard, a newly discovered protein, Skb1Hs, which may function as a chaperone or RAMP (receptor activity-modifying protein), has also been shown to interact with the rat SSTR1 C-tail [46].

Three types of protein kinases are known to phosphorylate GPCRs: second messenger-dependent kinases PKA and PKC; GRKs; and other kinases (e.g., casein kinase, tyrosine kinase) (reviewed in [17]). The fact that the inhibition or stimulation of PKA or PKC did not affect hSSTR1 up-regulation indicates that the obligatory phosphorylation of Ser^{360} is catalyzed by different kinases. Dextran sulfate and heparin, two inhibitors of GRKs [45], exert different effects. In the present study, dextran sulfate did not produce any change in SSTR1 up-regulation. On the other hand, heparin induced a small but significant reduction in SSTR1 up-regulation. These data strongly suggest that the activation of this kind of protein kinase is not essential. Although it has been shown that in SSTR1 stably transfected CHO-K1 cells, SST robustly activates the MAPK cascade [13], the specific inhibitor of MAPK kinase PD098,059 [13] did not have any effect on SST-induced hSSTR1 up-regulation. Significantly, treatment with KN-93, a broad-spectrum inhibitor of CaM protein kinases [46], completely abolished hSSTR1 up-regulation, suggesting a profound role of this type of protein kinase on receptor up-regulation. To our knowledge, we show here, for the first time, a relationship between CaM kinases and hSSTR up-regulation.

It has been described that for the thyrotropin-releasing hormone receptor to activate MAPK, the endocytic pathway must be intact, although the receptor itself does not need to undergo endocytosis [47]. However, sucrose or phenylarsine oxide, which both disrupt the endocytic machinery through clathrin coated pits, did not induce any changes in up-regulation, indicating that an intact endocytic pathway is not required for SST-induced hSSTR1 up-regulation. Furthermore, hSSTR1 up-regulation was partially inhibited by cholera toxin, and we have previously reported a similar effect with pertussis toxin [21]. These results imply that coupling to a second messenger system via G proteins is involved in up-regulation; however, neither the AC/PKA nor PLC/PKC pathways seem to be implicated. Although we cannot rule out the possibility that other G protein-activated second messenger pathways may also be involved in up-regulation, one possible mechanism to explain the effect of bacterial toxins is that there may be G proteins located in an intracellular location [48], such as the Golgi, that participate in hSSTR1 up-regulation in response to SST. Further supporting this hypothesis, brefeldin A, a disruptor of Golgi cisternae and inhibitor of trafficking from the Golgi to the cell surface [50], induced a more than 50% reduction in SST-induced up-regulation. Moreover, we have previously reported that hSSTR1 up-regulation in transfected CHO-K1 cells was unaffected by cycloheximide. Taken together, these results suggest that up-regulation is not due to new receptor synthesis but to the retargeting of an internal pool of pre-existing receptors to the plasma membrane [21]. The existence of an internal, inactive pool of SSTR1 has also been proposed in studies of agonist-induced phosphorylation of rat SSTR1 in stably transfected CHO-K1 cells [20]. A similar brefeldin A-sensitive/Golgi-localized pool of receptors has been described as being responsible for the recovery of the M_2 muscarinic cholinergic receptor to the cell surface after agonist treatment [49], and for protease-activated receptors (PARs) [51]. Consequently, these results and ours strongly suggest that a pool of pre-synthesized receptors in the Golgi zone is the main source of up-regulated hSSTR1.

We demonstrate here the essential participation of β-arrestins, particularly β-arrestin-1, in SST-induced hSSTR1 up-regulation. Classically, β-arrestin scaffold proteins were described to have a main function in rapid desensitization and internalization of GPCRs [15,17,18]. However, in the last few years, evidence has been accumulating to indicate that β-arrestins can also play important roles in triggering diverse intracellular signals, such as activation of MAPK cascades or photoreceptor apoptosis in *Drosophila* [17,18]. It has also been reported that the C-tails of β_2AR and

angiotensin II type 1A receptors contribute, depending on their relative capacity to form stable complexes with β-arrestin proteins, to the differential trafficking and regulation patterns of these receptors [51]. Here we present evidence for a new role of β-arrestins, namely, control of agonist-induced hSSTR1 up-regulation through interactions with the receptor's C-tail.

A surprising result of our study is that hSSTR1 up-regulation has proven to be a homologous phenomenon. Both biologically active forms of SST, and cortistatin, another naturally occurring peptide with strong similarities with SST [5], are able to trigger hSSTR1 up-regulation after long term treatment. However, a synthetic non-peptidic subtype selective analog for receptor 1 subtype [35] was unable to induce such a response. Indirect methods such as site-directed mutagenesis have proven that agonists interact with crucial noncontiguous residues in the extracellular and/or transmembrane domains of the receptor (reviewed in [2] and [3]). Moreover, in several studies of different peptide receptor systems, it has been demonstrated that small nonpeptide agonists interact with residues in the receptors differently from those that interact with the endogenous peptide agonist [3]. Therefore, we can hypothesize that the non-peptide analog binds to the receptor on a separate set of epitopes than SST, inducing conformational changes different from those that trigger up-regulation.

In conclusion, the results presented here demonstrate that hSSTR1 up-regulation after long-term agonist treatment is a homologous phenomenon, and is critically dependent on a specific ^{359}Lys-Ser-Arg^{361} sequence motif within the receptor's C-tail. Ser^{360} is an essential residue, further suggesting the participation of phosphorylation processes. hSSTR1 up-regulation requires interactions with β-arrestin-1, the participation of CaM protein kinases, and seems to be the result of the re-targeting of a pre-existing pool of receptors from the Golgi apparatus to the plasma membrane. On the other hand, coupling to AC and lack of internalization occur independently of molecular signals in the receptor's C-tail, although the latter process can also be regulated by β-arrestins.

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