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Distinct Phosphorylation Sites in the SST2A Somatostatin Receptor Control Internalization, Desensitization, and Arrestin Binding

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ABSTRACT

The somatostatin subtype 2A (sst2A) receptor, a member of the G protein-coupled receptor superfamily, mediates many of the neuroendocrine and neuromodulatory actions of somatostatin, and it represents the primary target for somatostatin analogs used in cancer therapy and tumor localization. Agonist stimulation leads to the rapid phosphorylation, endocytosis, and desensitization of the sst2A receptor; however, little is known about the role of phosphorylation in sst2A regulation. sst2A phosphorylation occurs on serine and threonine residues in the third intracellular loop and carboxyl terminus. Therefore, we generated mutant receptors in which serine (*Ser−*), threonine (*Thr−*), or both (*Ser−/Thr−*) residues in these regions were mutated to alanine. In contrast to the wild-type receptor, somatostatin treatment did not stimulate the phosphorylation of the *Ser−/Thr−* mutant, and it did not produce desensitization. Furthermore, internalization of the *Ser−/Thr−* mutant occurred 5 times more slowly than with the wild-type receptor. Mutating only the Ser residues did not inhibit either internalization or desensitization. In contrast, mutating only the Thr residues inhibited receptor endocytosis to the same extent as in the full mutant, but it did not affect receptor desensitization. In both the wild-type and *Ser−* receptors, agonist binding produced a stable arrestin-receptor complex that was maintained during receptor trafficking, whereas arrestin was not recruited to either the *Thr−* or the *Ser−/Thr−* receptors. These results demonstrate that agonist-stimulated receptor phosphorylation is necessary for both desensitization and rapid internalization of the sst2A receptor. However, sst2A receptor internalization and uncoupling can occur independently, involve different receptor phosphorylation sites, and exhibit different requirements for stable arrestin association.

G protein-coupled receptors (GPCRs) constitute the largest family of signaling proteins encoded by the human genome, and they affect a myriad of physiological and cellular events. Consistent with this critical role, GPCRs are highly regulated both by receptor availability at the cell surface and by changes in the efficiency with which available receptors are coupled to the signal transduction machinery. An important mechanism of GPCR regulation involves receptor phosphorylation by GRKs, a family of serine/threonine protein kinases that specifically phosphorylate receptors occupied by agonist (Gurevich and Gurevich, 2006; DeWire et al., 2007; Moore et al., 2007). According to the now classic model based largely on experiments with rhodopsin and the β-adrenergic receptor, phosphorylation of activated receptors by GRKs leads to the binding of cytoplasmic proteins called arrestins that act as adaptors to promote receptor association with components of the endocytic machinery and thus to stimulate receptor internalization. Receptor-bound arrestins also exhibit two signaling functions (Gurevich and Gurevich, 2006; DeWire et al., 2007; Moore et al., 2007). First, they prevent G protein activation by shielding their receptor partner from G proteins. Second, they act as scaffolds that recruit signaling molecules to their associated receptor and thus stimulate G protein-independent signaling. Accordingly, receptor uncoupling and endocytosis are both thought to result from arrestin binding to a GRK-phosphorylated, activated receptor (Gurevich and Gurevich, 2006; DeWire et al., 2007; Moore et al., 2007).

The cytoplasmic regions within GPCRs that are phosphorylated by GRKs and bind arrestins vary substantially among different receptors (Gurevich and Gurevich, 2006). Moreover,

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ABBREVIATIONS: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; sst2, somatostatin receptor subtype 2; CT, carboxyl terminus; IC3, third intracellular loop; PKC, protein kinase C; PPI, phosphatase inhibitors; SS14, the 14-amino acid form of somatostatin; HA, hemagglutinin; EGFP, enhanced green fluorescent protein; CHO, Chinese hamster ovary; PVDF, polyvinylidene difluoride; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; WT, wild type.

phosphorylation is not required for arrestin binding to all members of the GPCR family (Gurevich and Gurevich, 2006). To complicate matters further, in several GPCRs different intracellular receptor domains are required for desensitization and internalization (Pals-Rylaarsdam and Hosey, 1997; Huttenrauch et al., 2002; Celver et al., 2004; Barthet et al., 2005). Therefore, common structural elements for GPCR regulation have yet to be defined and the domains involved in receptor internalization and uncoupling cannot be readily predicted.

The neuroendocrine peptide somatostatin exerts its effects by binding to a family of five GPCRs (sst1–sst5) that are widely distributed in endocrine glands, the gastrointestinal tract, immune cells, and the central and peripheral nervous systems (Meyerhof, 1998; Olias et al., 2004; Schonbrunn, 2004). In addition, the sst2 receptor mRNA can undergo alternative splicing in some tissues to produce two receptor variants, sst2A and sst2B, that differ in their carboxyl termini (Cole and Schindler, 2000). Somatostatin acts as neuromodulator in the brain and spinal cord; as an inhibitor of hormone and exocrine secretion in the pituitary, pancreas, and gut; and as an inhibitor of intestinal motility. In addition, somatostatin controls cell proliferation and apoptosis in many types of tumors. In secretory cells, somatostatin receptors act primarily through $G_{i/o}$ proteins to inhibit adenylyl cyclase, open potassium channels, and inhibit voltage-activated calcium channels (Schonbrunn, 2004). However, sst receptors can affect a variety of other signal transduction pathways, including phospholipases C and A2 and tyrosine phosphatases (Lahlou et al., 2004; Olias et al., 2004; Schonbrunn, 2004). The sst2A receptor subtype is the most widely distributed somatostatin receptor in human tissues and in neuroendocrine tumors (Reubi et al., 1998; Reubi, 2003; Körner et al., 2005), and this receptor represents the primary molecular target for somatostatin analogs in cancer therapy and in their diagnostic use for tumor localization (Lamberts et al., 2002; Reubi, 2003).

The sst2A receptor undergoes rapid internalization and desensitization in a variety of differentiated cells (Golard and Siegelbaum, 1993; Stroh et al., 2000; Elberg et al., 2002) and in model cell lines (Hukovic et al., 1996; Hipkin et al., 1997; Liu et al., 2005). Agonists stimulate sst2A phosphorylation within minutes, and both the time course and the dose-response for somatostatin-induced receptor phosphorylation correlate closely with receptor endocytosis and desensitization (Hipkin et al., 1997; Elberg et al., 2002). In fact, sst2A receptor phosphorylation has even been observed in vivo, and it has been shown to correlate with receptor internalization (Liu et al., 2003). Because somatostatin analogs are so important in the clinical management of neuroendocrine tumors, elucidating the role and mechanism of receptor phosphorylation in sst2A function is critical for understanding the regulation of this receptor during therapy and under physiological conditions.

We previously showed that upon agonist stimulation, sst2A is phosphorylated exclusively in two receptor domains: the carboxyl terminus (CT) and the third intracellular loop (IC3) (Hipkin et al., 2000; Elberg et al., 2002). Phosphorylation occurs primarily on Ser residues and to a lesser extent on Thr (Hipkin et al., 1997). Although the sst2A receptor is phosphorylated by protein kinase C (PKC), agonist stimulated receptor phosphorylation is catalyzed by GRKs because 1) sst2A signaling is not required (Hipkin et al., 1997), 2) PKC inhibitors have no effect (Hipkin et al., 2000; Elberg et al., 2002), and 3) the activated receptor is efficiently phosphorylated by GRKs (Noble et al., 2003). Somatostatin treatment also leads to the rapid recruitment of arrestins to cell surface sst2A receptors and, as for other class B GPCRs (Oakley et al., 2000), arrestins remain associated with the receptor during intracellular vesicular trafficking to the perinuclear compartment (Brasselet et al., 2002; Tulipano et al., 2004; Liu et al., 2005). Although sst2A receptor phosphorylation, internalization, and desensitization all occur within minutes of agonist stimulation (Hipkin et al., 1997; Elberg et al., 2002), we do not know whether receptor phosphorylation is required for these regulatory processes. Moreover, the sst2A receptor domains important for internalization and uncoupling have not been characterized. Our goal was to identify the structural determinants for sst2A receptor regulation by determining the effect of phosphorylation site mutations on agonist-induced receptor internalization, desensitization, and arrestin binding. Our results demonstrate that sst2A phosphorylation is required for receptor endocytosis, uncoupling, and arrestin association; however, different phosphorylation sites are involved in these functions.

Materials and Methods

Reagents and Antibodies. Cell culture media and Geneticin (G-418) were purchased from Invitrogen (Carlsbad, CA). SS14 was purchased from Bachem California (Torrance, CA). Leupeptin, phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, bacitracin, Nonidet P-40, and pertussis toxin were obtained from Sigma-Aldrich (St. Louis, MO). *N*-Dodecyl-β-D-maltoside was purchased from Calbiochem (San Diego, CA). Phosphate-free Dulbecco's modified Eagle's medium and [^{32}P]orthophosphate were purchased from MP Biomedicals (Irvine, CA). Agarose-bound wheat germ agglutinin was purchased from Vector Laboratories (Burlingame, CA), and protein A/G PLUS-agarose was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Reagents for electrophoresis and the horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG were from Bio-Rad (Hercules, CA). Mouse monoclonal hemagglutinin (HA) antibody (HA.11) was purchased from Covance (Berkeley, CA), and rabbit polyclonal HA antibody was from Bethyl Laboratories (Montgomery, TX). 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) was purchased from Roche Diagnostics (Indianapolis, IN). Texas Red-labeled goat anti-mouse IgG was from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). SlowFade Light Antifade mounting solution was from Invitrogen. Oligonucleotide primers were synthesized by Sigma-Genosys (The Woodlands, TX), and the TOPO cloning kit was purchased from Invitrogen. All other reagents were of the best grade available, and they were purchased from common suppliers.

Plasmid Construction. Generation of the plasmid encoding wild-type rat sst2A receptor containing three tandem repeats of the HA epitope (YPYDVPDYA) at the amino terminus has been described previously (Liu et al., 2005). The receptor mutants with alanine substituted for serine and/or threonine residues were produced using either the transformer site-directed mutagenesis method (Clontech, Mountain View, CA) or the QuikChange kit (Stratagene, La Jolla, CA). The vector used for all constructs was pcDNA3.0 (Invitrogen), and all mutant receptors contained the same three tandem repeats of the HA epitope at the amino terminus as the wild-type receptor. Three mutants were generated (Fig. 1): 1) *Ser−* in which all serine residues in the third loop (Ser237, Ser238, Ser244, Ser245, and Ser250) and C terminus of sst2A (Ser316, Ser322, Ser333, Ser341, Ser343, Ser348, and Ser368) were mutated to alanine; 2) *Thr−* in which all threonine residues were mutated to

alanine, namely Thr353, Thr354, Thr356, Thr359, and Thr367; and 3) *Ser−/Thr−*, in which all the above-mentioned serine and threonine residues were mutated to alanine. The enhanced green fluorescent protein (EGFP)-β-arrestin-1 chimera was constructed by excising the rat β-arrestin-1 gene from a plasmid generously provided by Dr. Marc Caron (Duke University, Durham, NC) using SacI and KpnI. The gene was then inserted into the pEGFP-N3 Vector (Clontech), resulting in a hybrid protein with EGFP attached to the carboxyl terminus of β-arrestin-1. All constructs were sequenced to ensure accuracy.

Cell Culture. Stable clonal cell lines expressing wild-type or mutant sst2A receptors were generated by transfection of CHO-K1 cells with the appropriate sst2A pcDNA3 plasmid using FuGENE6 transfection reagent (Roche Diagnostics). After selection of stable transfectants with 750 μg/ml G-418 (Invitrogen), individual cell lines were isolated by dilutional cloning. Cloned cell lines were cultured in Ham's F-12 medium containing 10% fetal bovine serum and 250 μg/ml G-418 at 37°C and 5% CO_2. Experimental cultures were plated in medium without G-418 and used 2 to 3 days later.

Measurement of Receptor Phosphorylation. Metabolic labeling of cells and subsequent purification of the sst2A receptor was carried out essentially as described previously (Hipkin et al., 1997; Liu and Schonbrunn, 2001). In brief, CHO cells expressing HA-tagged sst2A receptors were incubated for 3 h with [^{32}P]orthophosphate in phosphate-free Dulbecco's modified Eagle's medium with 5 mg/ml lactalbumin hydrolysate (1 mCi of ^{32}P in 3.5 ml per 100-mm dish). SS14 (100 nM) was then added directly to the labeling medium, and the cells were incubated at 37°C under 5% CO_2 for an additional 15 min. After hormone stimulation, cells were scraped into ice-cold HEPES-buffered saline (150 mM NaCl, 20 mM HEPES, pH 7.4, 5 mM EDTA, and 3 mM EGTA) containing protease and phosphatase inhibitors (PPI: 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, 50 μg/ml bacitracin, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 0.1 mM sodium vanadate, and 100 nM okadaic acid). After centrifugation, cells were solubilized in lysis buffer (HEPES-buffered saline containing 4 mg/ml dodecyl-β-maltoside and PPI) for 60 min at 4°C, and they were centrifuged again at 100,000*g* for 30 min.

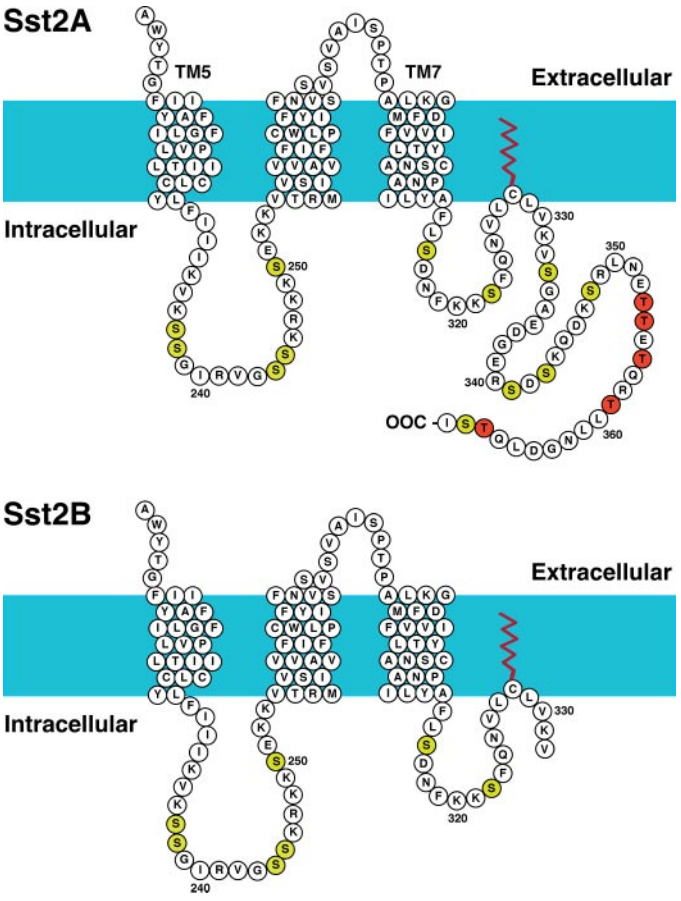

Fig. 1. Diagram of the potential phosphorylation sites in sst2 receptor splice variants. The structure of the carboxyl-terminal portion of the rat sst2A and sst2B receptors are shown schematically. The serine and threonine residues mutated to alanine in this study are indicated in yellow and red, respectively. We previously showed by phosphopeptide mapping that phosphorylation occurs on residues in the third intracellular and C-terminal regions of the sst2A receptor (Hipkin et al., 2000; Elberg et al., 2002).

Solubilized, radiolabeled receptors were subjected to a two-step purification consisting of lectin affinity chromatography followed by immunoprecipitation with mouse monoclonal HA antibody (Hipkin et al., 1997; Liu and Schonbrunn, 2001). For lectin affinity chromatography, the cell lysate was incubated at 4°C for 90 min or overnight with 100 μl (packed volume) of washed wheat germ agglutinin-agarose. After centrifugation, and extensive washing with lysis buffer, the adsorbed glycoproteins were eluted two times at 37°C for 30 min in lysis buffer containing 3 mM *N,N″,N‴*-triacetyl-chitotriose (Sigma-Aldrich), PPI, and 0.5% SDS (v/v). Eluted proteins were diluted 3-fold, and then they were incubated with a 1:500 dilution of mouse monoclonal HA antibody at 4°C overnight. Receptors were immunoprecipitated with protein AG PLUS-agarose at 4°C for 60 min, solubilized in sample buffer [62.5 mM Tris-HCl, 2% sodium dodecyl sulfate, 2% 2-mercaptoethanol (v/v), 6 M urea, and 20% glycerol, pH 6.8] at 60°C for 15 min, and then resolved on 10% sodium dodecyl sulfate-polyacrylamide gels. The resolved proteins were electrophoretically transferred to PVDF membrane, and then they were subjected to filmless autoradiographic analysis and autoradiography as described previously (Liu and Schonbrunn, 2001). The receptor concentration in each purified sample was determined by immunoblotting with rabbit anti-HA antibody as described previously (Liu and Schonbrunn, 2001), and the intensity of the receptor bands was quantitated using Scion Image version 1.63 (Scion Corporation, Frederick, MD). The amount of radiolabel in each receptor band was corrected for the immunoreactive receptor concentration in the same sample, and then it was expressed as a percentage of that found in the wild-type receptor.

For phosphoamino acid analysis, the piece of PVDF membrane containing the ^{32}P-labeled receptor was excised and incubated in 50 μl of 6 N HCl (Pierce Chemical, Rockford, IL) at 110°C for 2 h (Liu and Schonbrunn, 2001). Phosphoamino acids were resolved by two-dimensional thin layer electrophoresis on cellulose plates (Liu and Schonbrunn, 2001). Radioactivity comigrating with unlabeled phosphoamino acid standards was quantitated using a PhosphorImager (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Receptor Internalization. An enzyme-linked immunosorbent assay (ELISA) was used to measure the level of cell surface receptors in each clonal cell line and to quantitate the extent of receptor loss produced by internalization (Liu et al., 2005). Typically, cells were treated with SS14 at 37°C in Ham's F-12 medium containing 5 mg/ml lactalbumin hydrolysate and 20 mM HEPES, pH 7.4 (F12LH), for the times shown. The cells were then placed on ice, washed with ice-cold PBS, and fixed with 3% paraformaldehyde in PBS at room temperature for 10 min. After blocking with PBS containing 1% bovine serum albumin (fraction V) for 30 min at room temperature, cells were incubated with HA mouse monoclonal antibody at room temperature for 2 h. Cells were subsequently incubated with goat anti-mouse IgG horseradish peroxidase conjugate in blocking buffer at room temperature for 1 h, washed with PBS, and incubated with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) peroxidase substrate prepared according to the manufacturer's instructions. After 45 min at room temperature, 0.1-ml aliquots were transferred to a 96-well plate, and the optical density was measured at 405 nm in a microplate reader.

Confocal Immunofluorescence Microscopy. CHO cells expressing HA-tagged sst2A receptors were plated at a density of 200,000 cells/well onto 18-mm glass coverslips in 12-well plates. After 24 h, cells were transfected with 0.5 μg of EGFP-β-arrestin-1 using FuGENE 6 (Roche Diagnostics). Two days after transfection, cells were incubated with mouse anti-HA antibody (1:1000) for 2 h on ice in Ham's F-12 medium containing 5 mg/ml lactalbumin hydrolysate and 20 mM HEPES, pH 7.4, to label cell surface receptors. Cells were then incubated at 37°C without or with 100 nM SS14 for the indicated times. Hormone treatment was terminated by washing with ice-cold PBS, and cells were subsequently fixed with 3% paraformaldehyde in PBS for 20 min at room temperature. After washing again with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS at 4°C for 30 min, and then they were incubated with Texas Red-conjugated goat anti-mouse antibody (1:200; Jackson ImmunoResearch Laboratories Inc.) in PBS for 1 h at room temperature. After washing to remove unbound secondary antibody, coverslips were mounted using SlowFade mounting medium (Invitrogen). Confocal microscopy was performed on a laser-scanning confocal microscope (LSM 510; Carl Zeiss, Jena, Germany) in multitrack mode using excitation at 488 and 543 nm. EGFP and Texas Red were detected using band-pass filters from 500 to 530 nm and long-pass filters at 585 nm, respectively. Acquired images were processed using Photoshop (Adobe Systems, Mountain View, CA). Images are representative of the majority of the transfected cells at each time point. The results shown were observed in two to four independent experiments.

Desensitization of Adenylyl Cyclase Inhibition. Receptor desensitization was assessed by measuring changes in SS14 inhibition of adenylyl cyclase activity in membranes prepared from cells pretreated for 5 min in the absence or presence of 100 nM SS14. Cells were incubated in a CO_2 incubator at 37°C with 100 nM SS14 or carrier in serum-free medium containing 5 mg/ml lactalbumin hydrolysate. Pretreatment was stopped by washing the cultures with ice-cold HME buffer (20 mM HEPES, pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM benzamidine, 10 μg/ml soybean trypsin inhibitor, and 0.1 mg/ml bovine serum albumin). Washed cells were scraped into HME plus 10 μg/ml leupeptin, 20 mM tetrasodium pyrophosphate, and 0.1 μM okadaic acid, and then they were homogenized with a Dounce homogenizer. Homogenates were centrifuged on a step gradient of 23 and 43% sucrose in HE buffer (20 mM HEPES, pH 8.0, and 1 mM EDTA), and the fraction at the 23%:43% interface was collected and stored at −80°C. Membranes (5–10 μg protein/tube) were then assayed in triplicate for adenylyl cyclase activity at 30°C for 10 min as described previously (Liu and Schonbrunn, 2001). Reactions were performed in the presence of 10 μM forskolin and the indicated concentrations of SS14.

Data Analysis and Curve Fitting. Unless stated otherwise, results are expressed as the mean ± S.E.M. from replicate wells in a single experiment, and the results are representative of at least two independent experiments. Where not visible, error bars fell within symbol size. The half-time for agonist-induced receptor internalization was determined by fitting data to a single exponential decay curve using Prism version 4.0 (GraphPad Software Inc., San Diego, CA). Dose-response curves were analyzed by nonlinear regression fit of the data to a one-component sigmoidal curve with a Hill coefficient of 1. Statistical analysis of differences in EC_{50} values for control and SS14-pretreated groups was carried out with a ratio t test. Statistical analysis of differences in maximal inhibition was performed with a paired t test. Differences were considered significant at $P < 0.05$.

Results

Generation and Expression of sst2A Receptor Mutants. We previously showed that agonist treatment stimulates the rapid phosphorylation of the sst2A receptor on two domains: the IC3 and the CT (Hipkin et al., 2000; Elberg et al., 2002). To investigate the functional importance of receptor phosphorylation, we generated three mutant receptors with different Ser/Thr-to-Ala substitutions in these two receptor domains (Fig. 1): 1) *Ser−* in which all serine residues in the IC3 loop and the CT region were mutated to Ala, 2) *Thr−* in which all threonine residues in the CT region were mutated to Ala, and 3) *Ser−/Thr−* in which Ala was substituted for all serine and threonine residues in the IC3 loop and CT region. All receptor constructs contained a triple HA epitope tag at the amino terminus of the receptor to permit ready quantitation of plasma membrane receptors by ELISA (Liu et al., 2005). Addition of this extracellular epitope tag does not affect any of the functional properties of the receptor, including ligand binding affinity, receptor internalization, or inhibition of cyclic AMP production (Liu et al., 2005). All three sst2A receptor mutants were expressed at the cell surface to similar levels, responded to nanomolar concentrations of agonist, and coupled normally to inhibition of adenylyl cyclase (see below), demonstrating that the introduced mutations did not lead to general perturbations in receptor function.

Because both receptor internalization and signaling are known to be affected by the level of cell surface receptor expression (Cole et al., 2001; Dunigan et al., 2002), we screened multiple clonal cell lines stably expressing either wild-type or individual mutant receptors to identify clones that contained a similar density of cell surface receptors. Plasma membrane sst2A receptors were detected by ELISA with an antibody that recognized the amino-terminal HA epitope (Liu et al., 2005). Because our ELISA protocol was carried out with nonpermeabilized cells, the HA epitope was accessible to antibody in the medium only when the receptor was correctly expressed on the cell surface (Liu et al., 2005). In contrast, the R2-88 antibody, directed to the carboxyl-terminal region of sst2A (Gu and Schonbrunn, 1997), was unable to detect plasma membrane sst2A receptors in this ELISA unless the cells were first permeabilized with detergent (data not shown).

To quantitate receptor density, a standard curve of surface receptors as a function of cell protein was constructed for individual CHO-K1 cell clones expressing either the wild-type sst2A receptor (Fig. 2, top) or different mutant receptors (data not shown). Using a linear regression fit, we calculated the relative receptor density for each cell line, and then we selected cell clones that expressed similar levels of cell surface receptor (Fig. 2, bottom). In multiple independent experiments with the selected cell clones, the density of the *Ser−*, *Thr−*, and *Ser−/Thr−* mutant receptors were 126 ± 38% ($n = 3$), 152 ± 25% ($n = 5$), and 83 ± 19% ($n = 4$) that of the cell clone expressing wild-type receptors.

These results demonstrate that the wild-type and mutant sst2A receptors are expressed to similar levels in the selected CHO-K1 cell lines. The experiments described below show that all the receptors are also well coupled to adenylyl cyclase inhibition (Fig. 11; Table 1), confirming that the mutant receptors are present on the cell surface in a functional form.

Somatostatin Induced Phosphorylation of sst2A Receptor Mutants. Our previous peptide mapping experiments showed that the sst2A receptor was phosphorylated in the C-terminal and third intracellular loop domains after SS14 stimulation (Hipkin et al., 2000; Elberg et al., 2002). To determine whether receptor phosphorylation was reduced in the mutant

sst2A receptors as expected and to estimate the relative importance of Ser and Thr phosphorylation, we metabolically labeled cells with $^{32}PO_4$ and then we incubated them without or with 100 nM SS14 for 15 min. Receptors were then purified by lectin affinity chromatography followed by immunoprecipitation with an antibody to the amino-terminal HA epitope. The amount of radiolabel incorporated into purified receptors was quantitated using a PhosphorImager, and then it was corrected for receptor concentration as determined in the same samples by immunoblotting. Figure 3A shows the results of a representative experiment, and Fig. 3B summarizes the data from three independent experiments.

Basal phosphorylation was reduced in both the *Ser*− and

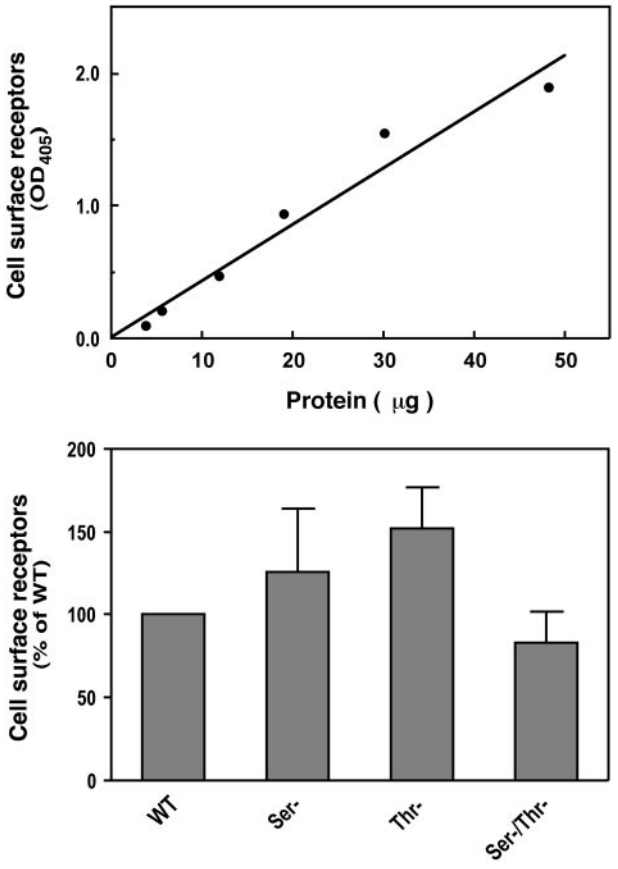

Fig. 2. Receptor density in cell lines expressing wild-type and mutant sst2A receptors. Top, a clonal CHO cell line expressing wild-type sst2A receptors was plated at different densities in 24-well plates. The next day, cells were incubated with HA antibody at room temperature for 2 h, washed to remove unbound antibody, and fixed with paraformaldehyde. Cell surface receptor was quantitated by ELISA as described under *Materials and Methods*. In replicate wells, cells were lysed with 0.1% Triton X-100, and the soluble protein was measured with the Bradford reagent. The graph shows the ELISA signal as a function of cell protein in duplicate wells. The error bars are within the symbol size. Surface receptors per microgram of cell protein were calculated by linear regression analysis. Bottom, surface receptor density was determined as shown in the top panel for each CHO cell line expressing a mutant sst2A receptor. For each clonal cell line, receptor density was expressed as a percentage of the density in the cell line expressing wild-type sst2A receptor. The results show the mean ± S.E.M. of three to five independent experiments, and they demonstrate that all four clonal cell lines express a similar density of cell surface receptors.

TABLE 1

Effect of SS14 on the internalization of wild-type and mutant sst2A receptors

Values for the EC_{50} and the extent of internalization for WT and mutant sst2A receptors were determined by nonlinear regression analysis of sigmoidal dose-response curves obtained in three independent experiments similar to that shown in Fig 4, bottom. Values for the half time of internalization were obtained from two to four independent experiments like the experiment shown in Figure 4, top. Data are presented as the mean ± S.E.M. for the EC_{50} and as the mean ± S.D. for $t_{1/2}$.

Receptor	EC_{50}	Maximal Internalization	$t_{1/2}$
	nM	*% of control*	*min*
WT	1.00 ± 0.12	77.5 ± 2.8	4.84 ± 0.76
Ser−	1.01 ± 0.06	98.9 ± 3.3	2.73 ± 0.28
Thr−	4.02 ± 0.99	49.6 ± 3.1	26.3 ± 3.7
Ser−/*Thr*−	2.89 ± 0.68	44.4 ± 10.4	41.3 ± 4.2

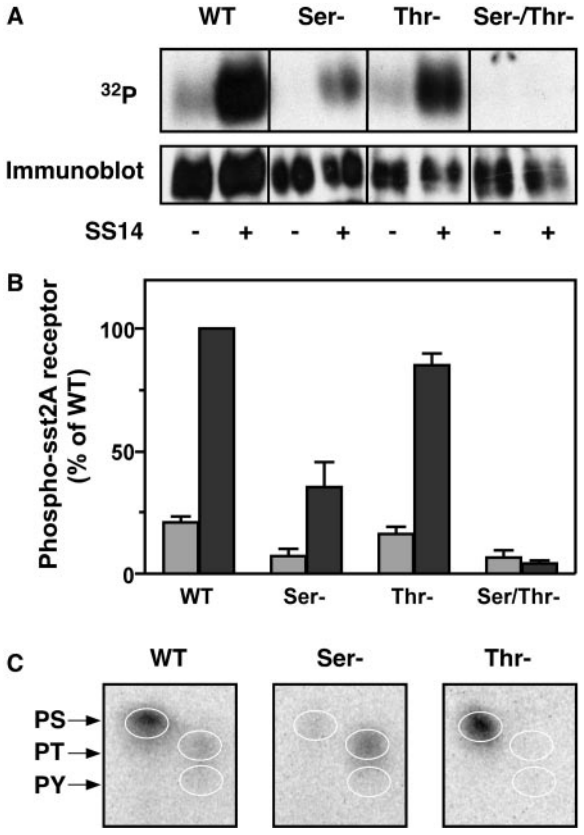

Fig. 3. SS14-induced phosphorylation of wild-type and mutant sst2A receptors. A, $^{32}PO_4$-labeled CHO cells expressing either wild-type, *Ser*−, *Thr*−, or *Ser*−/*Thr*− receptors were incubated in the absence or presence of 100 nM SS14 for 15 min. After detergent solubilization and incubation with wheat germ agglutinin-agarose, adsorbed glycoproteins were eluted with *N*, *N*′, *N*″′-triacetyl-chitotriose, and receptors were then immunoprecipitated with mouse anti-HA antibody. Immunoprecipitated proteins were subjected to SDS-polyacrylamide gel electrophoresis, and then they were electrophoretically transferred to PVDF membrane. The incorporation of radiolabel was visualized by autoradiography, and receptor density was determined by immunoblotting with rabbit anti-HA antibody. B, $^{32}PO_4$ incorporation into receptor bands was quantitated in the PVDF membranes with a PhosphorImager, and receptor density was measured in the immunoblots by densitometry using Scion Image. The amount of radiolabel in each receptor band was corrected for receptor concentration, and then it was expressed as a percentage of that found in the wild-type receptor. The graph shows the mean ± S.E.M. of ^{32}P incorporation into receptors isolated from untreated (▨) and SS14-treated (■) cells in three independent experiments. C, after electrophoretic transfer of proteins to PVDF membrane, phosphorylated receptors were located by autoradiography. The portions of the PVDF membrane containing receptor from SS14-treated cells were excised and incubated at 110°C with 6 N HCl for 2 h. The hydrolyzed samples were analyzed by two-dimensional thin layer chromatography in the presence of unlabeled carrier phosphoserine (PS), phosphothreonine (PT), and phosphotyrosine (PY) standards. The figure shows a phosphorimage of the thin layer chromatography plate. The migration of the unlabeled standards was determined by staining with ninhydrin and is shown by the circles. The results shown are representative of two independent experiments.

Thr− mutants, and it was completely eliminated in the *Ser*−/*Thr*− mutant. After SS14 stimulation, the amount of ^{32}P incorporated into the *Ser*− receptor was reduced by 65 ± 10% (mean ± S.E.M.; $n = 3$) compared with the wild-type receptor. However, radiolabeling was decreased by only 14.4 ± 3.0% in the *Thr*− mutant. Mutating both Ser and Thr residues abolished SS14-stimulated receptor phosphorylation. These results indicate that SS14 stimulates sst2A receptor phosphorylation on both Ser and Thr. However, the Ser residues constitute the major sites for sst2A receptor phosphorylation after agonist treatment.

The amino acids phosphorylated in the WT and mutant receptors were next characterized directly by phosphoamino acid analysis of purified receptors prepared from SS14-treated cells (Fig. 3C). Quantitation of the radiolabel incorporated into phosphoserine and phosphothreonine residues showed that in the wild-type receptor 80% of the ^{32}P was associated with phosphoserine and 20% was associated with phosphothreonine. In contrast, and as expected, ^{32}P was exclusively present in phosphoserine in the *Thr*− mutant, whereas phosphothreonine was labeled in the *Ser*− mutant. Although the acid hydrolysis method used in these experiments does not quantitatively convert all of the radioactivity present in a phosphoprotein to phosphoamino acids (Kamps, 1991), the results again show that SS14 stimulates sst2A receptor phosphorylation primarily on Ser residues.

In summary, the metabolic labeling experiments with $^{32}PO_4$ demonstrate that mutation of all the Ser and Thr residues in the C terminus and third intracellular loop of the sst2A receptor abrogates both basal and SS14-stimulated sst2A receptor phosphorylation, consistent with our previous peptide mapping data identifying these as the only phosphorylated domains in sst2A (Hipkin et al., 2000; Elberg et al., 2002). Serine residues constitute the primary sites for agonist-stimulated receptor phosphorylation, and threonine residues are phosphorylated to a much lesser extent.

Internalization of Wild-Type and Phosphorylation-Deficient Mutant Receptors. Figure 4 (top) shows the rate of internalization of wild-type and mutant sst2A receptors after stimulation with a saturating concentration (100 nM) of SS14. The calculated half-times determined in multiple independent experiments are summarized in Table 1. The *Ser*− receptor consistently demonstrated rapid internalization that was about 2 times faster than the wild-type receptor. In contrast, although the *Thr*− and *Ser*−/*Thr*− mutants still internalized after agonist stimulation, their rates of internalization were markedly reduced: the *Thr*− mutant receptor internalized about 5 times more slowly and the *Ser*−/*Thr*− mutant internalized about 8 times more slowly than wild-type sst2A (Table 1).

Figure 4 (bottom) shows the dose dependence for SS14-induced receptor internalization. A summary of the values for the EC_{50} and maximal internalization in three independent experiments is shown in Table 1. The *Ser*− mutant showed the same sensitivity to SS14 as the wild-type receptor, although the extent of *Ser*− receptor internalization was slightly higher. The *Thr*− and *Ser*−/*Thr*− mutants were 3- to 4-fold less sensitive to SS14-induced internalization, and the extent of receptor internalization at 30 min was less than that for the wild-type receptor.

The observation that SS14 stimulated receptor internalization with similar potencies in the WT and mutant receptors shows that the introduced mutations had minimal effects on agonist binding per se. Furthermore, the rapid internalization of the *Ser*− receptor demonstrates that phosphorylation of serine residues in the IC3 and CT domains is not essential. In contrast, the impaired rate of internalization of the *Thr*− and the *Ser*−/*Thr*− mutant receptors shows that phosphorylation of the threonine residues in the carboxyl-terminal domain is required for rapid sst2A receptor endocytosis.

***β*-Arrestin Trafficking in Cells Expressing Wild-Type and Phosphorylation-Deficient Mutant Receptors.** Arrestins have been shown to bind to many phosphor-

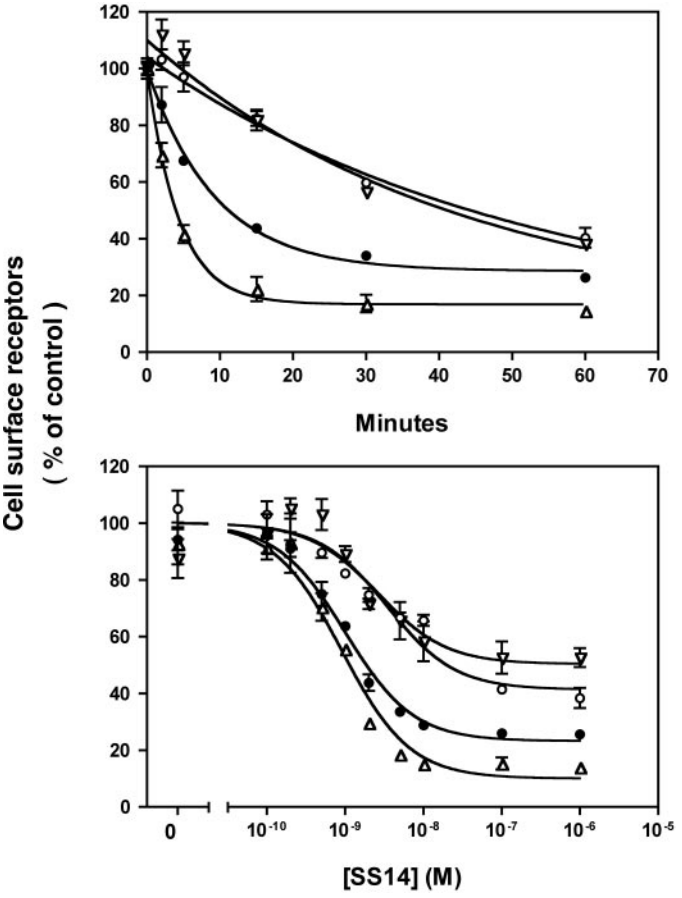

Fig. 4. Rate and dose-response for SS14-induced internalization of wild-type and phosphorylation-deficient sst2A receptors. Top, CHO cells expressing wild-type (●), *Ser*− (△), *Thr*− (▽), or *Ser*−/*Thr*− (○) sst2A receptors were incubated at 37°C for various times with 100 nM SS14. After washing at 4°C, the cells were fixed with paraformaldehyde, and cell surface receptors were measured by ELISA as described under *Materials and Methods*. Surface receptor levels at each time point were expressed as a percentage of the untreated control group, and the figure shows the results from a representative experiment with duplicate wells for each time point. The rate of receptor internalization followed first order kinetics as determined by nonlinear regression analysis. The fitted values for the half-time of internalization were 6.4 min for wild type, 3.0 min for *Ser*−, 29.9 min for *Thr*−, and 35.8 min for *Ser*−/*Thr*− receptors. The results from several independent experiments are summarized in Table 1. Bottom, CHO cells expressing wild-type (●), *Ser*− (△), *Thr*− (▽), or *Ser*−/*Thr*− (○) sst2A receptors were incubated at 37°C for 30 min with various concentrations of SS14. After washing at 4°C, cells were fixed with paraformaldehyde and surface receptors were measured by ELISA as described above. The results are expressed as a percentage of the untreated control group. Dose-response curves were calculated by nonlinear regression fit to a sigmoidal dose-response curve using GraphPad Prism version 4.0. The fitted values for the EC_{50} were 1.0, 0.9, 3.4, and 2.6 nM for wild-type, *Ser*−, *Thr*−, and *Ser*−/*Thr*− mutants, respectively. The results from three independent experiments are summarized in Table 1.

ylated, activated GPCRs and to mediate their internalization (Krupnick and Benovic, 1998; Gurevich and Gurevich, 2004). Indeed, we previously found that SS14 stimulation leads to the rapid translocation of β-arrestin2 to plasma membrane sst2A receptors and its subsequent internalization with the receptor into endocytic vesicles (Liu et al., 2005). Therefore, we determined whether differences in the internalization kinetics of wild-type and phosphorylation-deficient sst2A receptors reflected differences in arrestin recruitment.

Figures 5 to 8 show the cellular distribution of wild-type and phosphorylation-deficient sst2A receptors and EGFP-tagged β-arrestin1 after treatment with SS14. In the absence of agonist, wild-type receptors are localized to the plasma membrane and β-arrestin1-EGFP is uniformly distributed throughout the cytosol (Fig. 5). Two minutes after the addition of SS14, β-arrestin1 moves to the plasma membrane where it colocalizes with the receptor in small clusters around the cell periphery. With increasing time, the wild-type receptors traffic with β-arrestin1 into the cytosol where they occur together in punctate vesicles (t = 5 min) that subsequently cluster in the perinuclear region (t = 15 min). These results parallel those previously found with β-arrestin2 (Liu et al., 2005), and they demonstrate that the wild-type sst2A receptor associates tightly with both nonvisual arrestins.

SS14 stimulation of cells expressing the *Ser*− mutant produced a similar pattern of β-arrestin1 (Fig. 6) and β-arrestin2 (data not shown) trafficking. However, we noticed that β-arrestin translocation to the plasma membrane occurred somewhat more rapidly after SS14 treatment of cells expressing *Ser*− receptors compared with those expressing wild-type receptors, consistent with the faster internalization of this mutant than the wild-type receptor. Hence, maximal translocation of β-arrestin1 occurred after 1 min of SS14 treatment with the *Ser*− mutant (Fig. 6) and after 2 min with the WT receptor (Fig. 5).

In marked contrast, we were unable to detect either β-arrestin translocation to the plasma membrane or the association of β-arrestin with vesicular receptors in cells expressing the internalization-impaired sst2A mutants. Both the *Thr*− (Fig. 7) and the *Ser*−/*Thr*− (Fig. 8) receptors were endocytosed after SS14 stimulation, and they accumulated in cytoplasmic vesicles. However, β-arrestin1 remained uniformly distributed in the cytoplasm at all times—there was no recruitment to activated cell surface receptors or preferential association with internalized receptors in endocytic vesicles. Identical results were obtained when β-arrestin2-EGFP trafficking was monitored instead of β-arrestin1-EGFP (data not shown).

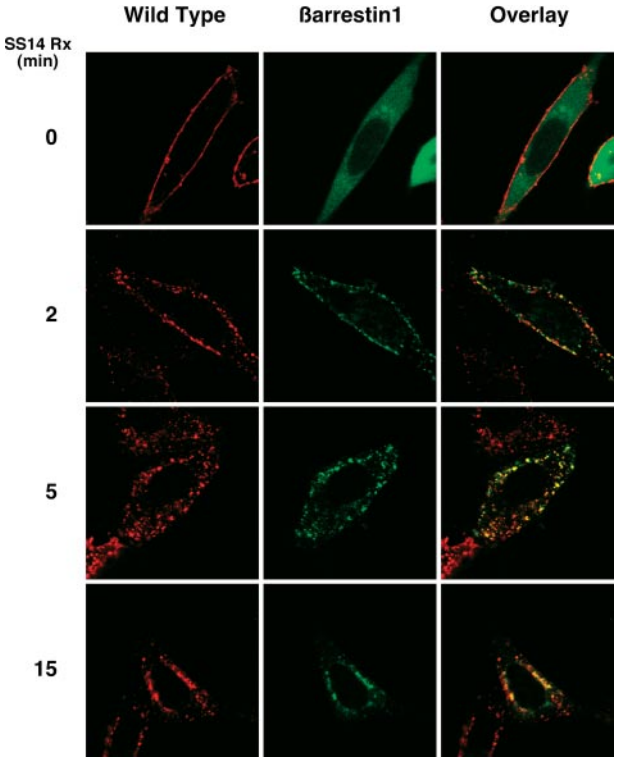

Fig. 5. β-Arrestin-1 translocation and cointernalization with the sst2A receptor after stimulation with SS14. CHO cells stably expressing wild-type sst2A receptor were transiently transfected with β-arrestin-1-EGFP as described under *Materials and Methods*. After 48 h, cells were incubated with HA antibody for 2 h on ice to label cell surface receptors. Cells were then treated with 100 nM SS14 at 37°C for the times shown, and subsequently they were fixed, permeabilized, and stained as described under *Materials and Methods*. Images were acquired with a confocal laser-scanning 510 microscope (Carl Zeiss). The same cells are shown at each time point, with green fluorescence indicating the distribution of β-arrestin-1 and red fluorescence showing the distribution of the sst2A receptor. The merged images show β-arrestin-1 and receptor colocalization. Images are representative of the majority of the transfected cells at each time point.

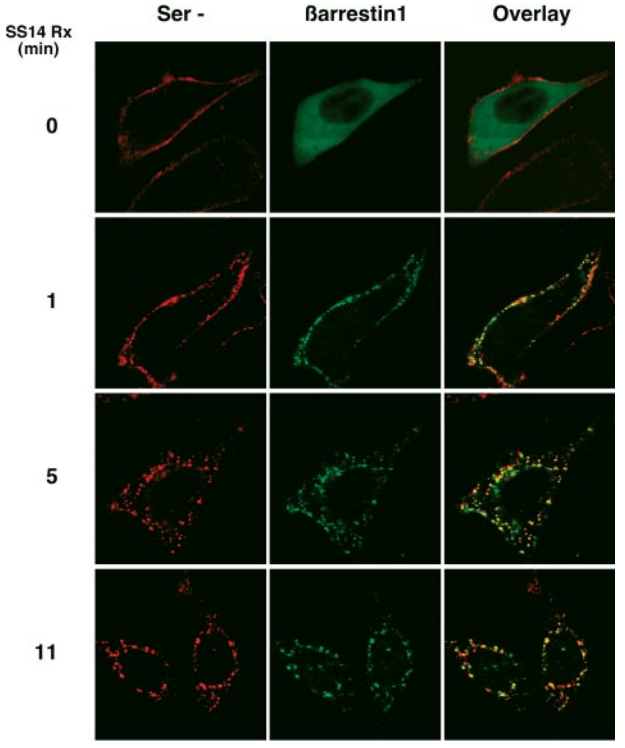

Fig. 6. β-Arrestin-1 translocation and cointernalization with the *Ser*− receptor after stimulation with SS14. CHO cells expressing the phosphorylation-deficient *Ser*− mutant of sst2A were transiently transfected with β-arrestin1-EGFP. After 48 h, cells were incubated with HA antibody on ice to label cell surface receptors. Cells were then treated with 100 nM SS14 at 37°C for the times shown, and subsequently they were stained and analyzed as described under *Materials and Methods.* The same cells are shown at each time point, with green fluorescence indicating the distribution of β-arrestin-1 and red fluorescence showing the distribution of the *Ser*− mutant sst2A receptor. The merged images show β-arrestin-1 and receptor colocalization. Images are representative of the majority of the transfected cells at each time point.

These findings demonstrate that the Thr residues in the C terminus of the sst2A receptor are required for stable interaction with β-arrestins, and they support the conclusion that Thr phosphorylation and arrestin recruitment are important for rapid receptor endocytosis. They further demonstrate that different sst2A receptor phosphorylation sites are not equally important for arrestin association: whereas mutation of the five Thr residues prevented the formation of a stable receptor-arrestin complex, mutation of the 12 Ser residues did not.

Effect of Sucrose and Pertussis Toxin on the Internalization of Wild-Type and Phosphorylation-Deficient Receptors. Because the rates of endocytosis of the Thr/Ala-substituted mutant receptors were markedly reduced, we next determined whether their internalization still occurred by a clathrin-mediated mechanism, as observed previously for the wild-type receptor (Hipkin et al., 2000). Cells were treated with 0.45 M sucrose for 30 min before SS14-induced internalization was carried out for 5 and 30 min (Fig. 9). Pretreatment with hypertonic sucrose, which has been shown to disrupt clathrin-coated pits (Heuser and Anderson, 1989), did not affect the density of cell surface receptors (t = 0). However, it blocked the SS14-induced internalization of all the mutant receptors and the wild-type receptor, indicating that receptor endocytosis occurred via a clathrin-mediated mechanism in all cases.

To assess the requirement for sst2A receptor $G_{i/o}$ coupling for receptor internalization, we preincubated cells in the absence or presence of 100 ng/ml pertussis toxin overnight, and then we measured the rate of receptor endocytosis after SS14 treatment. The data in Fig. 10 show that pertussis toxin did not inhibit the internalization of either the wild-type or *Ser−/Thr−* receptor. Therefore, neither $G_{i/o}$ activation nor the subsequent stimulation of second messenger-dependent kinases is required for SS14-induced sst2A receptor endocytosis.

Desensitization of Phosphorylation-Deficient sst2A Receptors. Because arrestin has been implicated in receptor desensitization as well as in receptor internalization, we next determined the extent to which SS14 pretreatment affected signaling by the phosphorylation-deficient sst2A mutants. To minimize the effect of receptor internalization on signaling, each clonal cell line was pretreated for only 5 min without or with 100 nM SS14, and then it was used to prepare membranes. As shown in Fig. 4, 5 min of SS14 exposure reduced cell surface receptors minimally with the *Thr−* and *Ser−/Thr−* mutants, and it produced only 10 and 30% loss of cell surface receptors with the WT and the *Ser−* mutants. The effect of SS14 on forskolin-stimulated adenylyl cyclase activity was measured in a subsequent challenge incubation. A representative experiment with each mutant is shown in Fig. 11, and Table 2 summarizes the results from multiple inde-

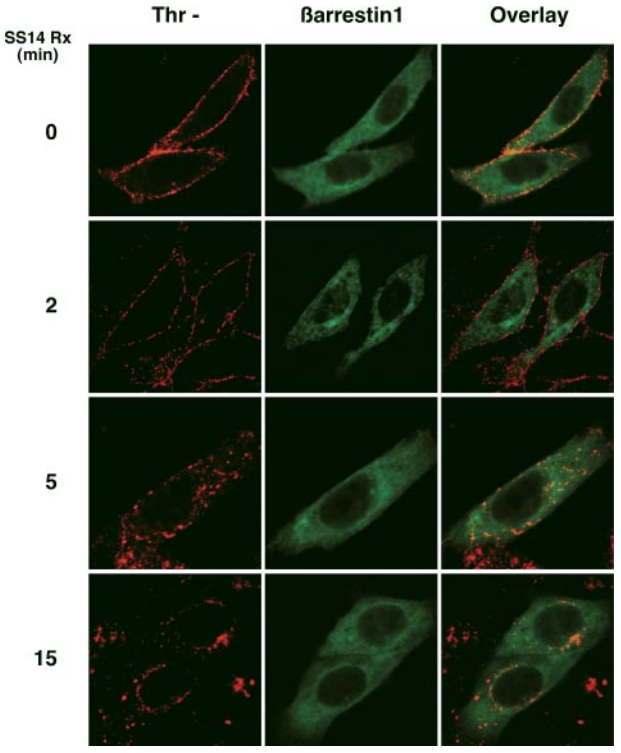

Fig. 7. β-Arrestin-1 does not traffic with the *Thr−* receptor after SS14 stimulation. CHO cells stably expressing the phosphorylation-deficient *Thr−* mutant of sst2A were transiently transfected with β-arrestin1-EGFP as described under *Materials and Methods.* Forty-eight hours after transfection, cells were incubated with HA antibody on ice to label cell surface receptors. Cells were then challenged with 100 nM SS14 at 37°C for the times shown, and subsequently they were stained and analyzed as described under *Materials and Methods.* The same cells are shown at each time point, with green fluorescence indicating the distribution of β-arrestin-1 and red fluorescence showing the distribution of the *Thr−* sst2A receptor mutant. Images are representative of the majority of the transfected cells at each time point.

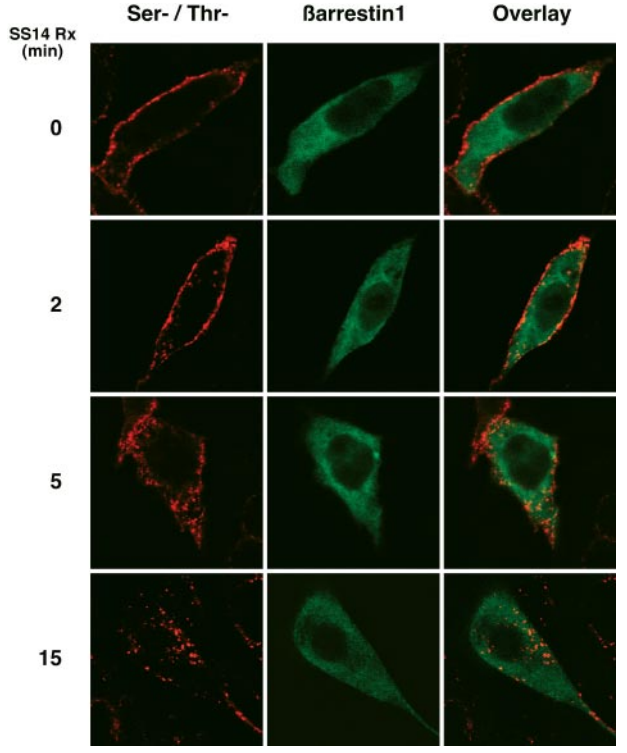

Fig. 8. β-Arrestin-1 does not traffic with the *Ser−/Thr−* receptor after SS14 stimulation. CHO cells stably expressing the phosphorylation-deficient *Ser−/Thr−* mutant of sst2A were transiently transfected with β-arrestin1-EGFP as described under *Materials and Methods.* Forty-eight hours after transfection, cells were incubated with HA antibody on ice to label cell surface receptors. Cells were then stimulated with 100 nM SS14 at 37°C for the indicated times, and subsequently they were stained and analyzed as described under *Materials and Methods.* The same cells are shown at each time point, with green fluorescence indicating the distribution of β-arrestin-1 and red fluorescence showing the distribution of the *Ser−/Thr−* sst2A receptor. Images are representative of the majority of the transfected cells at each time point.

pendent experiments. In all cells, SS14 inhibited adenylyl cyclase activity in nonpretreated cells with similar subnanomolar potencies. Therefore, all the mutant receptors were well coupled to adenylyl cyclase, and they had similar affinities for SS14.

SS14 pretreatment of cells expressing the wild-type receptor increased the EC_{50} value for SS14 inhibition of cyclase 12-fold, but it did not change the maximal inhibition produced (Fig. 11; Table 2). With the *Ser−* mutant, the EC_{50} value for SS14 was again significantly increased by pretreatment (5-fold), but in this case the maximal inhibition was also reduced by 40%. Pretreatment of cells expressing the *Thr−* receptors increased the EC_{50} values for SS14 12-fold but did not affect maximal inhibition, the same pattern as observed with the wild-type receptor. In contrast SS14 pretreatment of cells expressing *Ser−/Thr−* receptors had no effect on the subsequent responsiveness to SS14: neither the EC_{50} value for SS14 nor its maximal effect was altered.

These results show that the phosphorylation requirements for internalization and desensitization are different: whereas internalization was markedly inhibited in the *Thr−* receptor, desensitization was unaffected. In fact, because desensitization still occurred with both the *Ser−* and the *Thr−* mutants, phosphorylation at either sites is sufficient for receptor uncoupling. However, prevention of receptor phosphorylation at all sites in the *Ser−/Thr−* receptor completely blocked desensitization.

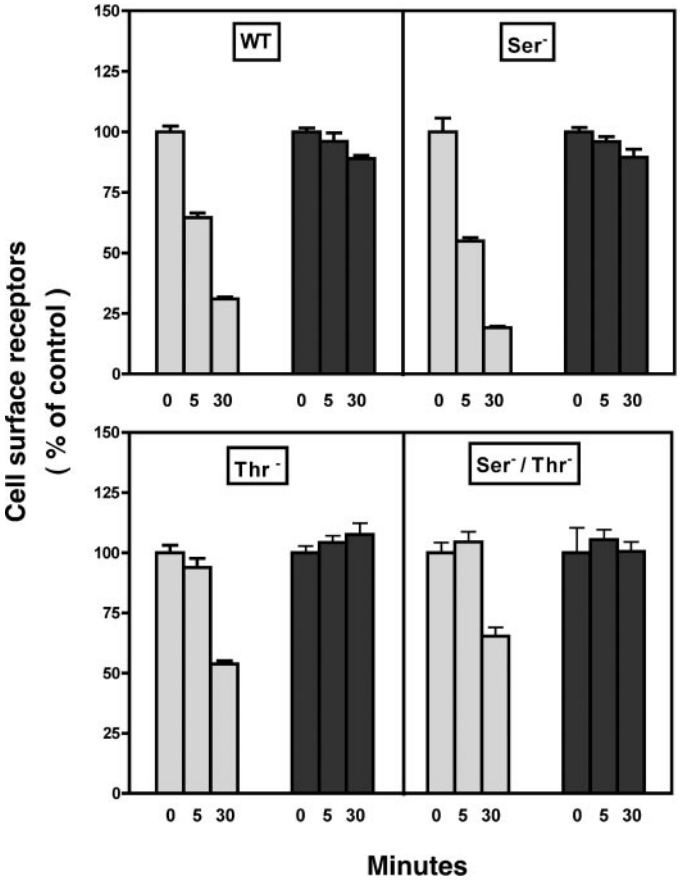

Fig. 9. Effect of sucrose on SS14 induced internalizaton of wild-type and phosphorylation-deficient mutants of the sst2A receptor. CHO cells expressing either wild-type, *Ser−*, *Thr−*, or *Ser−/Thr−* mutant receptors were preincubated for 30 min at 4°C without (▨) or with (■) 0.45 M sucrose. SS14 (100 nM) was then added (t = 0), and the cells were incubated further at 37°C in the continued absence or presence of sucrose for the times shown. After washing at 4°C, the cells were fixed with paraformaldehyde, and the receptors remaining at the cell surface were measured by ELISA. Surface receptor (mean ± S.E.M.) at each time point was calculated as a percentage of the control group at t = 0. Sucrose had no effect by itself on surface receptor density.

Discussion

The sst2A receptor undergoes desensitization and internalization within minutes of agonist stimulation. The major goal of this study was to test the importance of receptor phosphorylation and arrestin association in the desensitization and internalization of the sst2A receptor and to identify the receptor regions needed for these regulatory events. A related goal was to determine whether desensitization was caused by receptor endocytosis. Our results demonstrate that internalization and desensitization of the sst2A receptor are independent events with different requirements for specific receptor phosphorylation sites and for arrestin association.

Several intracellular domains can be phosphorylated in GPCRs upon agonist stimulation, and the importance of these different phosphorylated regions in the trafficking and desensitization of individual receptors is variable (Gurevich and Gurevich, 2006; DeWire et al., 2007; Moore et al., 2007). Peptide mapping has shown that SS14 stimulates the phosphorylation of the sst2A receptor on the CT and IC3 loop in pituitary (Hipkin et al., 2000), pancreatic acinar (Elberg et al., 2002), and CHO cells (Q. Liu and A. Schonbrunn, unpublished observations). Furthermore, Ser residues were phosphorylated to a much greater extent than Thr residues in GH pituitary cells (Hipkin et al., 1997). Consistent with our previous studies, we found that mutation of all the Ser/Thr residues in the CT and IC3 loop of sst2A prevents both basal

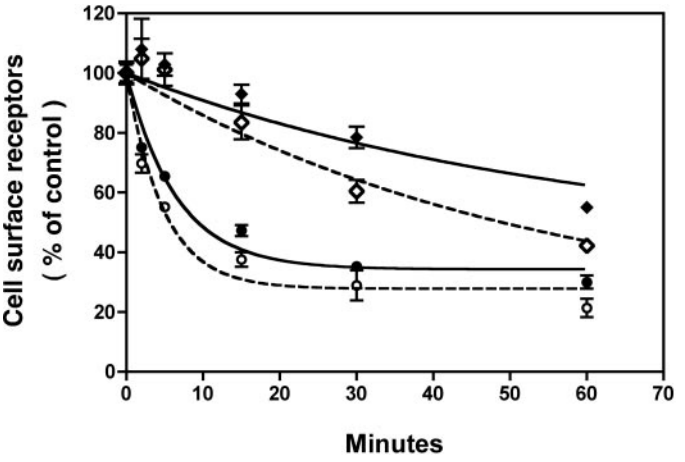

Fig. 10. Effect of pertussis toxin on SS14-induced internalization of wild-type and phosphorylation-deficient sst2A receptors. CHO-cells expressing either wild-type or *Ser−/Thr−* sst2A receptors were incubated overnight at 37°C without (●, ♦) or with (○, ◇) 100 ng/ml pertussis toxin. The rate of SS14-induced internalization of wild-type (●, ○) and *Ser−/Thr−* mutant (♦, ◇) sst2A receptors was then determined in the continued absence and presence of pertussis toxin as described in Fig. 3. Surface receptor (mean ± S.E.M.) at each time point was expressed as a percentage of the untreated control group. The rate of receptor internalization followed first order kinetics as determined by nonlinear regression analysis. The fitted values for the half-time of internalization without and with pertussis toxin treatment were 4.52 and 3.34 min for the wild-type receptor and 40.7 and 37.5 min for the *Ser−/Thr−* mutant receptor.

and SS14-stimulated receptor phosphorylation in CHO cells. Two new lines of evidence further indicate that receptor phosphorylation occurs mostly on Ser residues. First, phosphoamino acid analysis of the wild-type receptor isolated from SS14-treated cells showed 4 times as much ^{32}P incorporation into phosphoserine as into phosphothreonine. Second, phosphorylation was reduced to a much greater extent in the *Ser−* mutant than in the *Thr−* mutant. However, because partial mutation of receptor phosphorylation sites in the *Ser−* and *Thr−* mutants did not prevent agonist stimu-

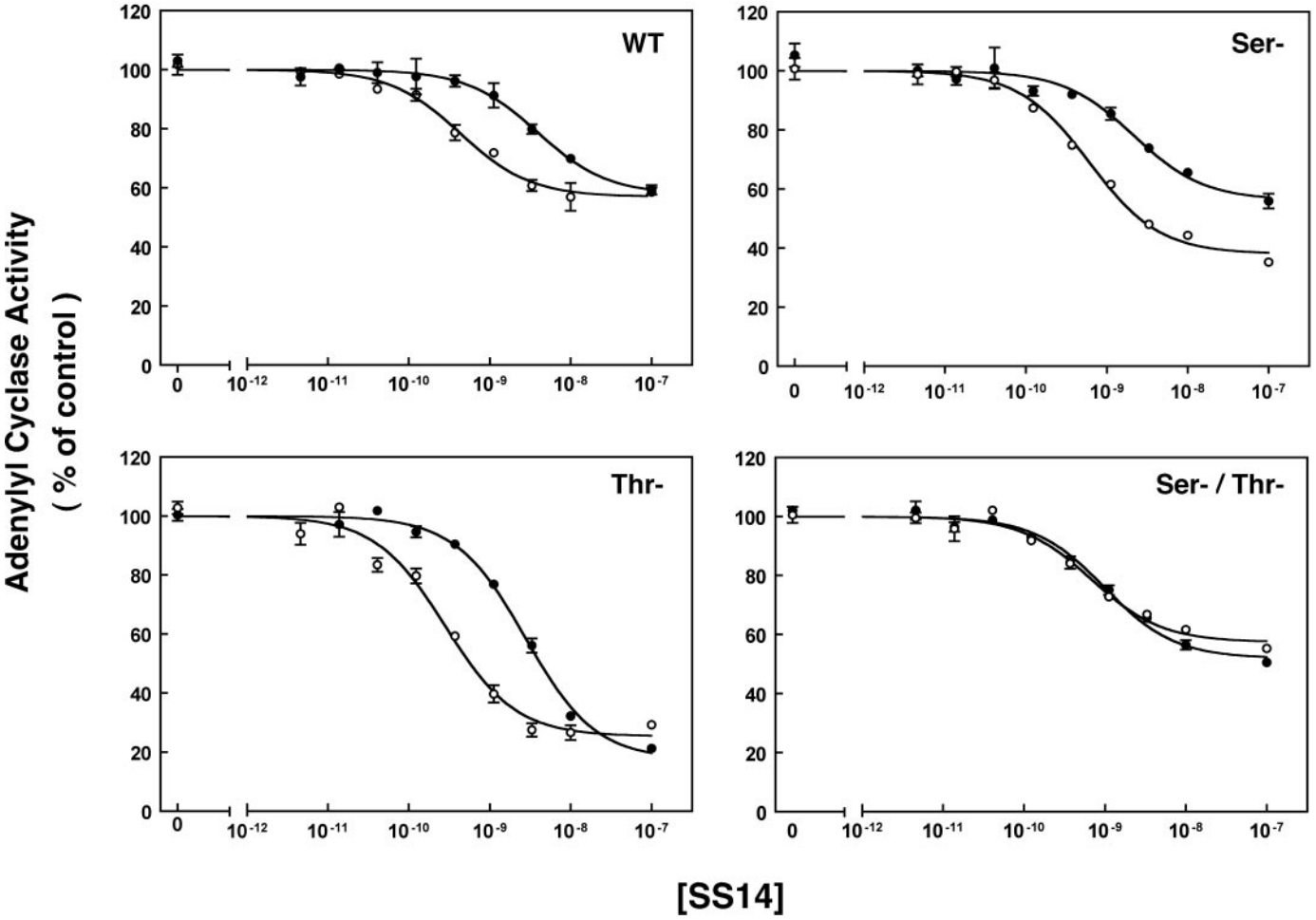

Fig. 11. Desensitization of SS14 inhibition of adenylyl cyclase in wild-type and phosphorylation-deficient mutants of the sst2A receptor. CHO cells expressing wild-type, *Ser−*, *Thr−*, or *Ser−/Thr−* mutant receptors were incubated without (○) or with (●) 100 nM SS14 at 37°C for 5 min, and then they were used to prepare membranes. Membrane adenylyl cyclase activity was measured as described under *Materials and Methods* in the presence of 10 μM forskolin and varying concentrations of SS14. Cyclase activity in each group is expressed as a percentage of the activity produced by stimulation with forskolin alone. Data are expressed as the mean ± S.E.M. of triplicate points determined in a representative experiment. Values for the EC_{50} and maximal cyclase inhibition were calculated for each experiment by nonlinear regression analysis of the dose-response curves. The results from several independent experiments are summarized in Table 2.

TABLE 2

Effect of SS14 pretreatment on the responsiveness of wild-type and mutant sst2A receptors

CHO cells expressing wild-type or mutant sst2A receptors were pretreated with 100 nM SS14 at 37°C for 5 min, and then they were used to prepare membranes. Adenylyl cyclase activity was determined as described under *Materials and Methods* in the presence of 10 μM forskolin and varying concentrations of SS14. Values for the EC_{50} and maximal inhibition were calculated for each experiment by nonlinear regression analysis of the sigmoidal dose-response curves as described in Fig 11. The data represent the mean ± S.E.M. values determined in *n* independent experiments. Statistical differences between the EC_{50} values for control and pretreated groups were determined by the ratio *t* test and are indicated as shown in the footnote. Differences in maximal inhibition were determined by a one-tailed *t*-test, and the test showed that maximal inhibition was significantly reduced by SS14 pretreatment only in the *Ser−* mutant ($P < 0.05$).

Receptor	EC_{50}		Maximal Inhibition		*n*
	Control	Pretreated	Control	Pretreated	
	nM		*% forskolin-stimulated activity*		
WT	0.34 ± 0.05	4.03 ± 1.05***	38.4 ± 4.7	35.4 ± 4.5	5
Ser−	0.38 ± 0.12	2.00 ± 0.08*	64.7 ± 1.2	39.3 ± 4.2*	3
Thr−	0.26 ± 0.04	3.23 ± 0.28**	68.7 ± 3.8	78.3 ± 3.2	3
Ser−/Thr−	0.61 ± 0.18	1.40 ± 0.41N.S.	42.8 ± 3.7	37.8 ± 5.2	6

N.S., no significant difference ($P > 0.05$).
* $P < 0.05$,
** $P < 0.01$,
*** $P < 0.001$.

lated phosphorylation on remaining residues, the regulation of these mutant receptors can be used to reveal the functional importance of the intact phosphorylation sites.

Desensitization of GPCRs, defined as a reduction in receptor signaling after pre-exposure to agonist, is a complex process that can involve either reduced coupling efficiency between cell surface receptors and the intracellular signaling machinery or a loss of plasma membrane receptors due to receptor endocytosis. Internalization and desensitization of the sst2A receptor both occur within a few minutes of SS14 treatment (Hipkin et al., 1997; Elberg et al., 2002). The simultaneous occurrence of these events makes it impossible to study receptor uncoupling in isolation. Nonetheless, we minimized the contribution of receptor trafficking to our desensitization assays by using a 5-min SS14 pretreatment and by measuring the subsequent receptor response in a cell-free assay in which receptor trafficking could not occur. In addition, because desensitization is often manifest as a change in EC_{50} instead of or in addition to a change in maximal inhibition, we carried out full dose-response curves for SS14 when testing for receptor uncoupling. Finally, we monitored the *rate* of receptor internalization rather than just the *extent* of internalization after a new steady-state was achieved. Together, these experimental paradigms, which differ from most previous studies of sst2A receptor regulation, were critical for quantitating receptor desensitization and endocytosis and for monitoring internalization and uncoupling separately in our internalization-deficient mutants.

The mutant receptors used in this study were all well expressed at the cell surface, and they inhibited adenylyl cyclase in response to nanomolar concentrations of SS14, demonstrating that the introduced mutations did not produce general changes in receptor function. Therefore, the fact that internalization was markedly slowed and receptor uncoupling was blocked in the phosphorylation negative *Ser−/Thr−* mutant indicates that sst2A receptor phosphorylation is required for both efficient endocytosis and uncoupling. However, different phosphorylated regions of the receptor were necessary for these regulatory events. Mutation of only Thr or only Ser residues did not prevent desensitization, although receptor phosphorylation was reduced in both cases. Thus, each group of residues can support receptor uncoupling, indicating that there is substantial structural flexibility in the desensitization mechanisms. In contrast, mutation of the Thr, but not the Ser residues, inhibited receptor internalization similarly to that seen with the full mutant, showing that the Thr residues alone are critical for efficient agonist induced receptor endocytosis despite the fact that Thr residues are phosphorylated to a much lesser extent than Ser residues.

Two comparisons demonstrate that receptor endocytosis and uncoupling are regulated independently. First, WT and *Thr−* receptors are desensitized similarly, even though the former is internalized 5 times more rapidly. Second, although the *Thr−* and the *Ser−/Thr−* mutant receptors internalize at a similar slow rate, the *Thr−* mutant is desensitized normally, whereas the *Ser−/Thr−* mutant does not desensitize. Interestingly, our conclusion that endocytosis and uncoupling of the sst2A receptor are independent events contrasts with the results obtained with the sst2B receptor, for which desensitization was found to be a consequence of receptor endocytosis (Beaumont et al., 1998). The sst2A and sst2B splice variants differ in the sequence and length of their C termini: the sst2B receptor ends after residue 332; therefore, it is missing five Ser and five Thr present in the C terminus of sst2A (Fig. 1). In fact, a recent report shows that although the sst2A receptor was rapidly phosphorylated upon agonist stimulation of colonic adenocarcinoma cells, phosphorylation of the sst2B receptor was not detectable (Holliday et al., 2007). The observed differences in the regulation of the two sst2 splice variants is likely to have important biological consequences in tissues, such as the brain and the gastrointestinal tract, in which alternative splicing of sst2 receptor mRNA occurs (Cole and Schindler, 2000).

Both uncoupling and internalization have been proposed to depend on the binding of arrestins to phosphorylated, activated GPCRs (Gurevich and Gurevich, 2006; DeWire et al., 2007; Moore et al., 2007). In fact, GPCRs have been classified on the basis of the affinity and duration of their association with arrestins after agonist activation and during the internalization process (Oakley et al., 2000). We previously showed that β-arrestin2 becomes tightly associated with the wild-type sst2A receptor after agonist stimulation (Liu et al., 2005). Here, we demonstrate that the same is true for β-arrestin1, in agreement with other studies (Brasselet et al., 2002; Tulipano et al., 2004). Thus, agonist treatment triggers the recruitment of both arrestins to the plasma membrane receptor, and the stable association of the receptor-arrestin complex is maintained during endocytosis and intracellular trafficking. The continuous association of the sst2A receptor with both arrestins during internalization shows that this receptor belongs to the class B GPCRs. Our new results with phosphorylation-deficient mutant receptors demonstrate that only the Thr residues in the C-terminal domain of the sst2A receptor are required for the formation of a stable arrestin-receptor complex: Ser residues in the C terminus and IC3 loop are dispensable, despite the fact that these Ser are heavily phosphorylated. Thus, arrestin binding to the sst2A receptor requires particular phosphorylated residues in the C terminus: these residues are not redundant or interchangeable.

The close correlation between the ability of different receptor mutants to internalize rapidly and their association with arrestins further indicates that arrestins have an important role in sst2A receptor endocytosis. However, internalization was not completely blocked in the *Thr−* and *Ser−/Thr−* receptors, which do not recruit arrestins; internalization continued at 10 to 20% the rate of the wild-type receptor. Thus, receptor phosphorylation is not an absolute requirement for sst2A internalization, but rather it greatly accelerates the process. These results suggest two mechanisms of sst2A receptor internalization: a rapid process, with a half-time of about 5 min that is dependent on receptor phosphorylation and stable arrestin association, and a slower process, with a half-time of 40 min, that still occurs with the nonphosphorylated *Ser−/Thr−* receptor mutant in the absence of arrestin recruitment. We do not know whether the trafficking pathways and mechanisms used for the internalization of the WT, *Thr−*, or *Ser−/Thr−* mutant receptors are the same or different. However, the ability of sucrose to block the endocytosis of both the *Thr−* and *Ser−/Thr−* mutant receptors as effectively as the wild-type receptor indicates that internalization always occurs by a clathrin-mediated pathway.

Whereas our results indicate that receptor-arrestin associ-

ation is required for efficient sst2A endocytosis, Brasselet et al. (2002) previously concluded that arrestins were not involved in the internalization of the sst2A receptor. The strongest support provided for this conclusion was an immunofluorescence experiment showing that the dominant-negative β-arrestin-1(319-418) did not block receptor internalization. However, our results suggest that the dominant-negative β-arrestin may only inhibit receptor endocytosis rather than prevent it completely. In our study, endocytosis of the slowly internalizing *Thr−* and *Ser/Thr−* mutants are both readily detected in similar immunofluorescence experiments, presumably because fluorescence microscopy is particularly sensitive to the intense signal produced by aggregated receptors in intracellular vesicles. Thus, the results from our studies and those of Brasselet et al. (2002) are not inconsistent once two rates of receptor endocytosis with different arrestin requirements are recognized. Overall, the data support the conclusion that rapid endocytosis of the sst2A receptor after agonist stimulation occurs by an arrestin-dependent mechanism, although the receptor can internalize at a slow rate in an arrestin-independent manner.

Unexpectedly, we found that the *Ser−* receptor internalized even more quickly than the WT receptor. Because the *Ser−* mutant was phosphorylated on Thr in response to SS14 stimulation, perhaps phosphorylation of the critical Thr residues in the C-terminal domain of sst2A is facilitated in the *Ser−* mutant, leading to faster receptor internalization. In fact, the observation that phosphorylation of the *Ser−* mutant is somewhat greater (35% of WT) than would be predicted from the phosphoamino acid analysis of the WT receptor (20% phosphothreonine) also suggests that Thr phosphorylation is enhanced in the *Ser−* mutant compared with the WT receptor. Clearly, site-specific measurements of receptor phosphorylation will be required to provide a molecular understanding of these intriguing observations.

In contrast to receptor internalization, the formation of a stable arrestin complex is not required for receptor uncoupling because the *Thr−* mutant, which did not recruit arrestin, desensitized as well as the WT receptor. This was a surprising result because Brasselet et al. (2002) had previously concluded that arrestin binding was essential for sst2A receptor desensitization from the observation that transiently transfected β-arrestin-1(319-418) blocked desensitization. However, the mechanism by which the truncated arrestin was able to block sst2A desensitization was not explained. β-Arrestin-1(319-418) represents the clathrin binding domain of arrestin, and although it binds to clathrin better than does native arrestin, it does not interact with phosphorylated GPCRs (Krupnick et al., 1997). Hence, it would not be expected to prevent the binding of endogenous arrestins to an activated receptor nor to interfere with arrestin-mediated desensitization. Without knowing the mechanism by which β-arrestin-1(319-418) was able to prevent sst2A receptor desensitization, the implication of such an effect is hard to evaluate. Our results show distinct requirements for receptor-arrestin association for sst2A receptor endocytosis and uncoupling: efficient internalization of different Ser/Thr substitution mutants parallels their ability to form a stable arrestin-receptor complex, whereas receptor uncoupling does not. The observation that receptor internalization and desensitization have different requirements for receptor-arrestin association raises the interesting question of how uncoupling occurs in the *Thr−* mutant.

In summary, the experiments described here provide the first detailed investigation of the role of receptor phosphorylation in the regulation of the sst2A somatostatin receptor. Our data show that endocytosis and uncoupling occur independently and that they exhibit a different arrestin dependence. Threonine residues in the CT region of sst2A are critical for stable arrestin association and efficient receptor endocytosis, but they are not required for receptor desensitization. In contrast, either Thr or Ser residues in sst2A can support receptor uncoupling. However, when all receptor phosphorylation sites are mutated, sst2A receptor desensitization is blocked. Therefore, phosphorylation plays a critical role in both the internalization and uncoupling of the sst2A receptor, but different phosphorylated regions are involved in these regulatory mechanisms, and they demonstrate a distinct dependence on stable arrestin binding.

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