Agonist-induced Desensitization, Internalization, and Phosphorylation of the sst2A Somatostatin Receptor*

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Cellular responsiveness to the inhibitory peptide somatostatin (SRIF) or its clinically used analogs can desensitize with agonist exposure. While desensitization of other seven-transmembrane domain receptors is mediated by receptor phosphorylation and/or internalization, the mechanisms mediating SRIF receptor (sst) desensitization are unknown. Therefore, we investigated the susceptibility of the sst2A receptor isotype to ligand-induced desensitization, internalization, and phosphorylation in GH-R2 cells, a clone of pituitary tumor cells overexpressing this receptor. A 30-min exposure of cells to either SRIF or the analog SMS 201–995 (SMS) reduced both the potency and efficacy of agonist inhibition of adenylyl cyclase. Internalization of receptor-bound ligand was rapid (t½ = 4 min) and temperature-dependent. SRIF and SMS increased the phosphorylation of the 71-kDa sst2A protein 25-fold within 15 min. Receptor phosphorylation was dependent on both the concentration and time of agonist exposure and was not affected by pertussis toxin pretreatment, indicating that receptor occupancy rather than second messenger formation was required. Receptor phosphorylation was also stimulated by phorbol 12-myristate 13-acetate activation of protein kinase C. Both ligand-stimulated and phorbol 12-myristate 13-acetate-stimulated receptor phosphorylation occurred primarily on serine. These studies are the first demonstration of agonist-dependent desensitization, internalization, and phosphorylation of the sst2A receptor and suggest that phosphorylation may mediate the homologous and heterologous regulation of this receptor.

The somatostatin peptides (SRIF-14 and SRIF-28) influence endocrine, exocrine, and neuronal function through binding to a family of six G protein-coupled receptors (sst1, sst2A, sst2B, sst3, sst4, and sst5) (1, 2). Within the SRIF receptor family, sst2A receptor mRNA has been detected in many tissues including the brain, pituitary, pancreas, spleen, small intestine, and stomach (1, 2), and the receptor protein has recently been shown to be widely distributed in the mammalian brain (3). Thus, this receptor isotype mediates many of the central and peripheral actions of SRIF.

Early studies on the signal transduction mechanisms activated by SRIF showed that sst receptors elicited their actions predominantly via pertussis toxin-sensitive G proteins (1, 2, 4). Thus, SRIF inhibition of adenylyl cyclase and Ca2+ channels, as well as SRIF stimulation of K+ channels, phospholipase C, serine/threonine and tyrosine phosphatases, arachidonic acid release, and mitogen-activated protein kinases are inhibited by pertussis toxin treatment (5–12). However, some actions of SRIF, such as stimulation of other tyrosine phosphatases as well as inhibition of Na/H exchange, are pertussis toxin-insensitive (13, 14). The network of signaling pathways activated by individual sst receptor isotypes is largely unknown. Signaling mechanisms have been especially difficult to elucidate in the native environment of the receptors because most SRIF target cells express multiple sst receptor isotypes that cannot be individually activated with the analogs currently available.

For most G protein-coupled receptors, hormone treatment decreases receptor responsiveness (desensitization), receptor levels (down-regulation), or both. However, relatively little is known about sst receptor regulation, especially following acute hormonal challenge. Exposure to SRIF or to selective SRIF agonists such as SMS 201–995 (SMS) has been reported to lead to desensitization in pituitary cells over the course of hours, days, or weeks (15–18). Desensitization occurring over both hours (19, 20) and minutes (21) has been reported in the AtT20 corticotrophic pituitary cell line, depending on the signaling pathway being examined. However, SRIF receptor desensitization was not detected in the mammotrophic GH4C1 pituitary cell line (22) and does not occur during long term treatment of many human pituitary tumors with SMS (23). SRIF receptors can be either down-regulated (24) or up-regulated (22) by hormone pretreatment depending on the cell type examined. The SRIF receptor isotypes involved in these varying effects are unknown although sst2 receptors are expressed, along with other sst receptor subtypes, in the normal pituitary, in pituitary tumors, and in the AtT20 and GH4C1 cell lines (2, 25).

Interestingly, for sst2A receptors exogenously expressed in Chinese hamster ovary cells, receptor binding has been reported to be either decreased (27, 28) or increased (29) by SRIF pretreatment. However, no desensitization studies have been reported with this receptor isotype.

Ligand-dependent and -independent desensitization of other G protein-coupled receptors is mediated by receptor phosphorylation (30). Examination of sst2A receptor phosphorylation

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1 The abbreviations used are: SRIF, somatostatin; SMS, SMS 201–995 (o-Phe-Cys-Phe-Gly-Trp-Lys-Thr-Cys-Thr-OH); VIP, vasoactive intestinal peptide; PMA, phorbol 12-myristate 13-acetate; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; GRK, G protein-coupled receptor kinase; PBS, phosphate-buffered saline.

2 Y.-Z. Gu and A. Schonbrunn, Mol. Endocrinol., in press.
requires a cell line expressing high levels of functional receptor protein. However, identification of appropriate cell lines for studies of exogenously expressed sst2A receptors has proven problematic. Although it is well established that SRIF inhibits adenyl cyclase activity in native cells (1, 2, 4), the coupling of the sst2A receptor to adenyl cyclase in heterologous cell lines varies with the cell model (1) suggesting that components required for faithful mimicry of the normal function of the sst2A receptor are not ubiquitously expressed. The GH4C1 pituitary tumor cell line, which contains both the sst1 and sst2A receptors as the parental GH4C1 cells and has both the elevated receptor expression required for phosphorylation studies and an environment that allows normal receptor coupling. By taking advantage of the 10,000-fold greater affinity of SMS 201–995 for the sst2 over the sst1 receptor (1), we now demonstrate agonist-dependent desensitization, internalization, and phosphorylation of the sst2A receptor in this pituitary model cell line.

**EXPERIMENTAL PROCEDURES**

**Hormones and Supplies**—Cell culture medium and G418 were purchased from Life Technologies, Inc. The sst2A-receptor antisera (R2-88) has been described.5 Leupeptin, pepstatin A, phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, bacitracin, cholesterol hemisuccinate, Nonidet P-40, and Protein A were obtained from Sigma. N-Dodecyl β-maltoside and pertussis toxin were purchased from Calbiochem and List Biological Laboratories, Inc. (Campbell, CA), respectively. CNBr-activated Sepharose 4B was from Pharmacia Biotech Inc. (Upsala, Sweden). Bradford reagent and reagents for electrophoresis and Western blotting were obtained from Bio-Rad. Carrier-free Na[221]I was purchased from Amersham Corp. Phosphate-free Dulbecco's modified Eagle's medium and [32P]orthophosphate were purchased from ICN Pharmaceuticals and [3H]-dodecyl sulfate-polyacrylamide gel. Bradford reagent and reagents for electrophoresis and Western blotting were obtained from Bio-Rad. Carrier-free Na[221]I was purchased from Amersham Corp. Phosphate-free Dulbecco's modified Eagle's medium and [32P]orthophosphate were purchased from ICN Biomedicals (Irvine, CA). All other reagents were of the best grade available and purchased from common suppliers.

**Cell Culture**—The clonal GH-R2.20 cell line (hereafter referred to as GHR2 cells) was generated by transfecting GH4C1 pituitary tumor cell line, which contains both the sst1 and sst2A receptors as the parental GH4C1 cells and has both the elevated receptor expression required for phosphorylation studies and an environment that allows normal receptor coupling. By taking advantage of the 10,000-fold greater affinity of SMS 201–995 for the sst2 over the sst1 receptor (1), we now demonstrate agonist-dependent desensitization, internalization, and phosphorylation of the sst2A receptor in this pituitary model cell line.

**Phosphorylation of the sst2A Receptor**

**Radioligand Binding and Internalization**—[Leu6,Trp22,Tyr25]-somatostatin-28 (Bachem California, Torrance, CA) and the sst2 receptor-selective somatostatin analog (36) (Sandoz Pharmaceuticals) were radioiodinated using chloramine T and subsequently purified by reverse-phase high performance liquid chromatography as described previously (33). GH-R2 cells were incubated at 4 °C in 1 ml of binding buffer (F10 medium containing 20 mM HEPES and 5 mM l-alanyl-lactalbumin hydrolysat, pH 7.4) containing approximately 100,000 cpm of [125I]-Tyr3-SMS (Sandoz Pharmaceuticals) with or without unlabeled SRIF (34). After 60 min, the cells were rinsed to remove unbound tracer and then incubated in fresh 37 °C buffer to allow internalization of the receptor-bound ligand. Cells were subsequently incubated on ice for 5 min in acidic glycine-bUFFER (100 mM glycine, 50 mM NaCl, pH 3.0) to release surface-bound ligand. After collection of the acidic buffer, the cells were dissolved in 0.1 N NaOH. The radioactivity in both glycine buffer (representing surface-bound ligand) and in the cell lysates (representing internalized ligand) was measured (34). Specific binding was calculated as the difference between the amount of radioligand bound in each fraction in the absence (total binding) and presence of 100 nM SRIF (nonspecific binding).

**Detection of sst2A Receptor by Immunoblotting**—GH-R2 cells were washed, scraped into cold PBS, pelleted, and solubilized in PBS containing 4 mg/ml dodecyl β-maltoside, 200 μg/ml cholesterol hemisuccinate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, and 50 μg/ml bacitracin for 60 min at 4 °C. The detergent lysates were clarified by centrifugation at 100,000 × g for 30 min, and the protein content of the supernatants was assessed by the method of Bradford (35). Protein was dissolved in sample buffer (62.5 mM Tris-HCl, 2% sodium dodecyl sulfate, 10% 2-mercaptoethanol, 6 μm urea, 20% glycerol, pH 6.8) by incubation at 60 °C for 15 min prior to resolution on a 7.5% sodium dodecyl sulfate-polyacrylamide gel.

Resolved proteins were transferred to PVDF membrane as described previously. The membrane was then blocked for 2 h with “Blotto” (10 mM NaH2PO4, 0.1% nonfat dry milk, 0.2% Tween 20) and incubated overnight at 4 °C with 1:20,000 dilution of anti-sst2A antibody R2-88. Following repeated washing, the membrane was incubated with 1:5000 dilution of goat-anti-rabbit antibody conjugated with horseradish peroxidase at room temperature for 1 h. Immunoreactive proteins were detected with the ECL chemiluminescent antibody detection system (Amersham Corp.).

**Detection of Immunoprecipitated sst2A Receptor**—GH-R2 cells were incubated in growth medium in the presence or absence of protein kinase activators for 30 min. Cell membranes were prepared as described above and solubilized by agitation at 4 °C for 60 min in HEPES-buffered saline (150 mM NaCl, 20 mM Heps, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, 50 μg/ml bacitracin, 5 mM EDTA, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 0.1 mM sodium orthovanadate) containing 4 mg/ml dodecyl β-maltoside, 200 μg/ml cholesterol hemisuccinate (lysis buffer). After centrifugation at 100,000 × g for 30 min, the supernatant was incubated overnight at 4 °C with anti-sst2A-receptor antisera covalently coupled to Protein A-Sepharose (final dilution of 1:200). The immunoprecipitated proteins were eluted from the Sepharose beads without mercaptoethanol for 15 min at 60 °C. After removal of the Sepharose beads and the addition of 10% mercaptoethanol (v/v), proteins were resolved on 7.5% sodium dodecyl sulfate-polyacrylamide gels, transferred to PVDF membrane, and immunoblotted with anti-sst2A antisera as described above.

**Purification of the Phosphorylated sst2A Receptor**—Metabolic labeling of cells and subsequent immunoprecipitation of the sst2A receptor was carried out as described previously (36, 37). Briefly, cells (1 dish/treatment) were incubated for 3 h in 3.5 ml of phosphate-free Dulbecco's modified Eagle's medium containing 1 μCi of [32P]orthophosphate and either 1% newborn calf serum or 5 mg/ml l-alanyl-lactalbumin hydrolysate. Hormones and pharmacological agents were then added directly to the labeling medium, and the cells were further incubated at 37 °C under 5% CO2 for the indicated times. The cells were then scraped into cold Heps-buffered saline, pelleted, and solubilized in lysis buffer containing phosphatase inhibitors for 60 min at 4 °C. The detergent lysates were centrifuged at 100,000 × g for 30 min, and the protein content of the supernatants was assessed by the method of Bradford (35).

The sst2A receptors were subjected to a two-step purification consisting of lectin affinity chromatography followed by immunoprecipitation with receptor antibody (36). Briefly, cell lysate protein (2 mg/ml) were incubated at 4 °C for 90 min with 100 μl (packed volume) of washed wheat germ agglutinin-agarose (Vector Laboratories, Inc., Burlingame, CA). Following centrifugation, the washed wheat germ agglutinin-agarose was washed vigorously with 30 volumes of lysis buffer, and adsorbed glycoproteins were eluted at 4 °C for 90 min with 250 μl of lysis buffer containing 3 mM N-acetylglucosamine and 0.5 mM N-acetylglucosamine bisectoisitol cholate.
(Sigma) and 0.5% SDS (v/v). Eluted proteins were diluted 5-fold and incubated with a 1:200 dilution of the anti-sst2A receptor antibody R2-88 at 4 °C for 90 min. The samples were then incubated at 4 °C for 90 min with 25 μl (packed volume) of protein A-Sepharose. Following centrifugation, the beads were washed as described previously, and the immunoprecipitated proteins were solubilized in sample buffer (60 °C, 15 min) and resolved on 7.5% sodium dodecyl sulfate-polyacrylamide gels.

For phosphoamino acid analysis, SDS-PAGE-resolved proteins were transferred to PVDF membrane, and the piece of membrane containing the 32P-labeled receptor (detected by autoradiography) was excised and incubated in 100 μl of 5.7% HCl (Pierce) at 110 °C for 0.5 or 2 h (37). Phosphoamino acids were resolved by two-dimensional thin layer electrophoresis on cellulose plates (37). Receptor phosphorylation and phosphoamino acid analyses were quantitated using a PhosphorImager (37).

Other Methods—Protein A (Sigma) was covalently coupled to CNBr-activated Sepharose B according to the manufacturer’s instructions (Pharmacia). Antireceptor IgG was covalently coupled to protein A-Sepharose as described previously. Photoaffinity labeling of the membrane sst2A receptor with [Leu6,p-Trp22,125I-Tyr25]somatostatin-28 and N-5’-azido-2’-nitrobenzoyl-N-oxysuccinimide (Pierce) and subsequent immunoprecipitation of the receptor with anti-sst2A was accomplished by published procedures (38). Unless otherwise indicated results of a representative experiment are shown. All experiments were repeated at least 2 times.

RESULTS

Desensitization and Internalization of sst2A Receptor Expressed in GH-R2 Cells—Whereas GH4C1 cells contain low levels (~0.1 pmol/mg of cell protein) of a mixture of the somatostatin receptor subtypes sst1 and sst2 (25, 39), the transfected GH-R2 cell line expresses approximately 10 pmol of the sst2A receptor/mg of protein (data not shown). Incubation of intact GH-R2 cells for 2 h at 4 °C with the sst2 selective ligand [125I-Tyr3]SMS and increasing concentrations of unlabeled peptides showed dose-dependent inhibition of radioligand binding with both SRIF (EC50 = 2.24 ± 0.31 nM) and SMS (EC50 = 38.9 ± 7.5 nM). The relative affinities of the rat sst2A receptor for these two ligands thus agrees well with those of the human sst2A receptor as determined in membrane binding studies (40).

To test the susceptibility of the sst2A receptor to desensitization, GH-R2 cells were incubated in the absence or presence of 100 nM SMS for 30 min at 37 °C. Membranes were then prepared, and the effect of pretreatment on hormonal regulation of adenyl cyclase activity was determined (Fig. 1). SMS pretreatment did not affect either basal or VIP-stimulated adenyl cyclase activity (data not shown). In membranes from untreated cells SMS inhibited VIP-stimulated adenyl cyclase activity with an EC50 of 1.2 ± 0.1 nM. Maximal inhibition was 63.7 ± 1.5%. Preincubation of cells with SMS attenuated both the potency (EC50 = 7.0 ± 0.4 nM) and efficacy of SMS inhibition (maximum inhibition = 36.2 ± 2.3%). Treatment of cells with 100 nM SRIF for 30 min had the same effect as SMS; maximal inhibition was reduced from 60.1 ± 2.0 to 41.2 ± 2.6% while the EC50 for SMS was increased from 1.0 ± 0.2 to 6.9 ± 2.5 nM. Therefore, exposure to agonist results in homologous desensitization of the sst2A receptor.

To ascertain if peptide binding induced rapid internalization of the ligand-receptor complex, cells were incubated for 1 h at 4 °C with [125I-Tyr3]SMS to occupy cell surface receptors, washed to remove unbound peptide, and then warmed to 37 °C for different periods of time (Fig. 2). A rapid, time-dependent internalization of the receptor-bound ligand occurred at 37 °C reaching a steady state by 90 min (Fig. 2, upper panel). The rate of internalization was fit to the sum of two first order reactions, giving a value of 4.0 ± 0.7 min for the half-time of internalization of the receptor-ligand complex (Fig. 2, lower panel). These results show that following binding to cell surface sst2A receptors, the bound ligand is rapidly internalized in a temperature-dependent manner.

Immunodetection of sst2A Receptor in GH-R2 Cells—To determine whether the sst2A receptor was phosphorylated, we next developed and validated methods for the detection and purification of the sst2A receptor protein. The immunoblot in Fig. 3 (left panel) shows that a receptor antibody that specifically recognizes the sst2A receptor isotype reacted with a broad 71-kDa protein band in GH-R2 cell extracts. This band was not detected in immunoblots incubated with preimmune sera or with immune sera in the presence of 1 μM antigen peptide. To determine whether this 71-kDa protein was the sst2A receptor, GH-R2 membranes were photoaffinity-labeled with [Leu6,p-Trp22,125I-Tyr25]SRIF-28 and N-5’-azido-2’-nitrobenzoyl-N-oxysuccinimide in the presence or absence of 100 nM SRIF (38). Membranes were then either directly solubilized with sample buffer (Fig. 3, middle panel) or solubilized with a non-denaturing detergent and then immunoprecipitated with preimmune or immune sst2A receptor antisera (Fig. 3, right panel). Subsequent analysis by SDS-PAGE and autoradiography showed that a 71-kDa protein was photoaffinity-labeled in membranes and that radiolabeling was effectively competed with SRIF, as expected for a high affinity sst receptor (middle panel). The photoaffinity-labeled protein was immunoprecipitated by receptor antisera but not by preimmune antisera nor by immune serum in the presence of 1 μM antigen peptide (right panel). Therefore, the receptor antibody effectively precipitated the 71-kDa sst2A receptor protein expressed in GH-R2 cells.

Agonist-induced Phosphorylation of sst2A Receptor in Intact Cells—To determine if agonist binding stimulated sst2A receptor phosphorylation, GH-R2 cells were labeled with [32P]orthophosphate and incubated in the absence or presence of

\[ \frac{\text{VIP-stimulated adenyl cyclase activity}}{\text{} \mu \text{C} \times \text{min}^{-1} \times \text{mg}^{-1} \text{protein}} \]
This receptor aggregate appeared to be generated during the and then solubilized in SDS sample buffer under reducing labeled (data not shown) receptors were immunoprecipitated with SRIF increased the phosphorylation of the 71-kDa sst2A although it is difficult to discern in Fig. 4. Treatment of cells of basal receptor phosphorylation was detectable with long film and analyzed by SDS-PAGE and autoradiography. A low level sst2A receptor was immunoprecipitated with receptor antibody partial purification by lectin affinity chromatography, the sample buffer with 6M urea to dissociate receptor aggregates. to solubilize the immunoprecipitated receptor by heating in antigen peptide (data not shown). Interestingly, it was necessary immune serum or with immune serum in the presence of an-...tion data were fitted to the equation $B_t/B_0 = Ae^{-kt} + (1 - Ae^{-kt})$ using KaleidaGraph$^{TM}$ 3.0 and gave the following fitted values: $A = 0.584$, $k_1 = 0.213$ min$^{-1}$, and $k_2 = 0.00123$ min$^{-1}$, where $A$ is the fraction of receptor dissociating with a rate constant of $k_2$ and $(1 - A)$ is the fraction of receptor dissociating with a rate constant of $k_2$. 100 nm SRIF for 15 min. Following detergent solubilization and partial purification by lectin affinity chromatography, the sst2A receptor was immunoprecipitated with receptor antibody and analyzed by SDS-PAGE and autoradiography. A low level of basal receptor phosphorylation was detectable with long film exposure (Figs. 6 and 8) or by analysis with a PhosphorImager, although it is difficult to discern in Fig. 4. Treatment of cells with SRIF increased the phosphorylation of the 71-kDa sst2A receptor protein 22 ± 6-fold over basal ($n = 5$). The 71-kDa phosphoprotein was not immunoprecipitated with either pre-immune serum or with immune serum in the presence of antigen peptide (data not shown). Interestingly, it was necessary to solubilize the immunoprecipitated receptor by heating in sample buffer, with 6 M urea to dissociate receptor aggregates. When either phosphorylated (Fig. 4, lane 3) or photoaffinity-labeled (data not shown) receptors were immunoprecipitated and then solubilized in SDS sample buffer under reducing conditions but without urea and heating, a higher molecular weight band was observed in addition to the 71-kDa receptor. This receptor aggregate appeared to be generated during the immunoprecipitation procedure since it was not present in GH-R2 cell extracts as analyzed by immunoblotting (Fig. 3, left panel) or in solubilized photoaffinity-labeled membranes (data not shown).

If sst2A receptor phosphorylation plays a role in either agonist-stimulated sst2A receptor desensitization or internalization, phosphorylation should be increased within the time frame of these regulatory events. As shown in Fig. 5, stimulation of sst2A receptor phosphorylation was half-maximal after a 2-min incubation with 100 nM SRIF, was maximal by 5 min, and was then maintained for at least 30 min. SMS also increased receptor phosphorylation by 2 min (data not shown) and was as effective as SRIF (Fig. 5).

Agonist stimulation of sst2A receptor phosphorylation was also concentration-dependent (Fig. 6). Phosphorylation of the sst2A receptor was significantly elevated by 3 nM SRIF and was further increased with higher doses of SRIF up to a maximal effect with 100 nM peptide. Incubation of $^{32}$P-labeled GH-R2 cells with concentrations of SRIF greater than 100 nM did not produce any additional stimulation (data not shown). Thus, phosphorylation of the sst2A receptor increases in response to ligand stimulation in a time- and concentration-dependent manner.

To assess the importance of sst2A receptor-Ge/o coupling for SRIF-induced receptor phosphorylation, GH-R2 cells were pre-treated with 100 ng/ml pertussis toxin for 24 h. This treatment abolished SRIF inhibition of VIP-stimulated cAMP accumulation (data not shown). However, SRIF-induced phosphorylation of the sst2A receptor was unaffected by pertussis toxin pretreatment (Fig. 7). Therefore, functional interaction of the sst2A receptor with pertussis toxin-sensitive G proteins is not necessary for agonist-induced sst2A receptor phosphorylation.

GH$_4$C$_1$ rat pituitary cells express m$_2$ muscarinic and A$_1$-adenosine receptors that couple to the same pertussis toxin-sensitive effectors pathways as SRIF (41). However, incubation of $^{32}$P-labeled GH-R2 cells with either the muscarinic agonist...
incubation with the Ca²⁺ stimulation of cAMP synthesis with forskolin had no effect. a 32-fold increase in sst2A receptor phosphorylation whereas incubation with the protein kinase C activator PMA stimulated

regulated protein kinases on sst2A receptor phosphorylation, To assess the effect of second messenger-cascades is not sufficient to stimulate sst2A receptor phosphorylation. Thus heterologous activation of SRIF-stimulated second messenger cascades is not sufficient to stimulate sst2A receptor phosphorylation.

The Effect of Protein Kinase Activators on sst2A Receptor Phosphorylation—To assess the effect of second messenger-regulated protein kinases on sst2A receptor phosphorylation, [32P]-labeled GH-R2 cells were incubated for 15-min with either no additions (Control), 100 nM SRIF, 200 nM phorbol 12-myristate 13-acetate (PMA), or 10 μM forskolin prior to sst2A receptor purification. As shown in Fig. 8 (bottom panel), a 15 min incubation with the protein kinase C activator PMA stimulated a 32-fold increase in sst2A receptor phosphorylation whereas stimulation of cAMP synthesis with forskolin had no effect. Incubation with the Ca²⁺ ionophore ionomycin (10 μM for 15 min) also did not alter sst2A receptor phosphorylation (data not shown). Therefore, heterologous activation of protein kinase C increased sst2A receptor phosphorylation whereas stimulation of either protein kinase A or Ca²⁺-dependent protein kinases apparently did not.

The signal intensity observed following immunoprecipitation of the [32P]-labeled receptor is determined by 1) the stoichiometry of receptor phosphorylation and 2) the receptor concentration. The latter depends, in turn, on receptor immunoprecipitation efficiency. The sst2A antibody used in this study recognizes a region in the sst2A receptor cytoplasmic tail containing potential phosphorylation sites. Hence phosphorylation in this region could decrease the efficiency with which the sst2A receptor is immunoprecipitated and thereby prevent the detection of [32P] labeling as was shown to occur with the bombesin receptor (37). To test whether receptor phosphorylation affected the ability of the antibody to recognize the receptor protein, GH-R2 cells were incubated for 15 min with no additions or with either 100 nM SRIF, 200 nM PMA, or 10 μM forskolin. Membranes were then prepared, and the ability of the antibody to recognize the different phosphorylated forms of the receptor was assessed both by immunoblotting (Fig. 9, upper panel) and by immunoprecipitation (Fig. 9, lower panel). Incubation of GH-R2 cells with SRIF, PMA, or forskolin for 15 min did not affect cellular sst2A receptor levels and did not alter the immunoprecipitation efficiency of sst2A receptor (Fig. 9). Therefore, the [32P] incorporation into the purified receptor (Figs. 3–8) accurately reflects its phosphorylation state.

Phosphoamino Acid Analysis of Phosphorylated sst2A—To identify the phosphorylated residues in the sst2A receptor, phosphoamino acid analysis was carried out with receptor from

Fig. 4. The effect of SRIF on sst2A receptor phosphorylation. [32P] labeled GH-R2 cells were incubated in the absence or presence of 100 nM SRIF for 15 min. Following detergent solubilization and partial purification by lectin chromatography, the sst2A receptor was immunoprecipitated with receptor antiserum at a final dilution of 1:200. Immunoprecipitated proteins were solubilized either in sample buffer containing 6 M urea for 15 min at 60 °C or in the same sample buffer without urea for 15 min at room temperature and then analyzed by SDS-PAGE and autoradiography.

Fig. 5. Time course for SRIF stimulation of sst2A receptor phosphorylation. [32P]-labeled GH-R2 cells were incubated either with 100 nM SRIF for the times shown or with 100 nM SMS 201–995 for 30 min. Following detergent solubilization and partial purification by lectin chromatography, the sst2A receptor was immunoprecipitated with receptor antiserum at a final dilution of 1:200. Immunoprecipitated proteins were analyzed by SDS-PAGE and either autoradiography (gel inset) or phosphorimaging (graph).

Fig. 6. Concentration dependence for SRIF stimulation of sst2A receptor phosphorylation. [32P]-labeled GH-R2 cells were incubated with the indicated concentration of SRIF for 30 min. Following detergent solubilization and partial purification by lectin chromatography, the sst2A receptor was immunoprecipitated with receptor antiserum at a final dilution of 1:200. Immunoprecipitated proteins were analyzed by SDS-PAGE and either autoradiography (gel inset) or phosphorimaging (graph).

Fig. 7. The effect of pertussis-toxin pretreatment on SRIF stimulation of sst2A receptor phosphorylation. GH-R2 cells were incubated in the absence or presence of 100 ng/ml pertussis toxin (PTX) for 24 h prior to and during cell labeling with [32P]orthophosphate. [32P]-labeled cells were then incubated in the absence or presence of 100 nM SRIF for 15 min. Following detergent solubilization and lectin chromatography, the sst2A receptor was immunoprecipitated with receptor antiserum at a final dilution of 1:200. Immunoprecipitated proteins were analyzed by SDS-PAGE and autoradiography.
Phosphorylation of the sst2A Receptor

Control cells and from cells incubated with either SRIF or PMA. Following acid hydrolysis of the receptor for 30 min, phosphoserine and phosphothreonine residues were detected (Fig. 10, top panel). Hydrolysis for 2 h facilitated detection of phosphoserine and phosphothreonine residues (Fig. 10, bottom panel). Under all three treatment conditions, the most heavily labeled residue was phosphoserine. However, all three phosphoamino acid species were detectable following analysis of sst2A receptor from unstimulated cells, and phosphorylation of all three residues was increased in a concentration-dependent manner in response to agonist stimulation (data not shown). These studies show that basal and SRIF- and PMA-stimulated sst2A receptor phosphorylation occur primarily on serine residues, although an increase in the phosphorylation of threonine and tyrosine also occurs.

**DISCUSSION**

The studies presented here demonstrate for the first time that hormone binding leads to rapid desensitization, internalization, and phosphorylation of the sst2A receptor.

Desensitization to native SRIF peptides as well as to the clinically used analog SMS or octreotide has been observed in some tissues and cancers but not in others, suggesting that susceptibility to desensitization depends on the receptor subtypes present. For example, in GH4C1 cells, which express both the sst1 and sst2 receptors (4, 25), prolonged exposure to SRIF does not attenuate subsequent SRIF inhibition of adenylyl cyclase activity following pretreatment of cells with sst5 receptor mRNA is not expressed in GH-R2 cells (26). The studies presented here demonstrate for the first time that hormone binding leads to rapid desensitization, internalization, and phosphorylation of the sst2A receptor. Following acid hydrolysis of the receptor for 30 min, phosphoserine and phosphothreonine residues were detected (Fig. 10, top panel). Hydrolysis for 2 h facilitated detection of phosphoserine and phosphothreonine residues (Fig. 10, bottom panel). Under all three treatment conditions, the most heavily labeled residue was phosphoserine. However, all three phosphoamino acid species were detectable following analysis of sst2A receptor from unstimulated cells, and phosphorylation of all three residues was increased in a concentration-dependent manner in response to agonist stimulation (data not shown). These studies show that basal and SRIF- and PMA-stimulated sst2A receptor phosphorylation occur primarily on serine residues, although an increase in the phosphorylation of threonine and tyrosine also occurs.

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Phosphorylation of the sst2A Receptor

As in the case of desensitization, most studies of SRIF receptor internalization have utilized cell lines expressing multiple sst receptors whose composite behavior was monitored using SRIF analogs that bound to several sst receptor isotypes. Hormone binding did not lead to receptor-mediated internalization in either GH3 pituitary cells or in RINm5F insulinoma cells (34, 43). In contrast, variable amounts of receptor-bound peptide were internalized in AtT-20 pituitary cells, human pituitary tumor cells, islet cells, and pancreatic acinar cells (44–47). In these early studies the internalization of the hormone-receptor complex could not be attributed to a specific sst receptor subtype. We show here that agonist binding to the sst2A receptor triggers rapid receptor-mediated internalization. Very recently the sst2 receptor was also reported to mediate internalization of bound [Leu8,p-Trp22,125I-Tyr25]SRIF-28 when expressed in Chinese hamster ovary cells (29). However, in Chinese hamster ovary cells only about 20% of the receptor-bound ligand was internalized even after 60 min. The explanation for the differences in the extent of receptor-mediated internalization in the two studies is not clear; internalization of the receptor-ligand complex could be influenced by both the cellular environment and the nature of the bound ligand.

The mechanisms mediating sst2A receptor desensitization and internalization are not known but, as has been postulated for other G-protein coupled receptors, may involve receptor phosphorylation (30, 48, 49). Indeed, this hypothesis is consistent with our findings that 1) agonist stimulation of sst2A receptor phosphorylation (t½ ~ 2 min, Fig. 5) occurs concurrently with receptor internalization in GH-R2 cells (t½ ~ 4 min, Fig. 2) and that 2) maximal sst2A receptor phosphorylation is evident under conditions used to elicit sst2A receptor desensitization.

Phosphorylation of other G protein-coupled receptors is catalyzed by two types of kinases: second messenger-activated kinases and G protein-coupled receptor kinases (GRKs). Our results suggest that agonist-stimulated phosphorylation of the sst2A receptor preferentially involves GRKs. SRIF inhibition of adenyl cyclase, as well as regulation of other signaling pathways, is blocked by pertussis toxin, which prevents coupling of sst receptors to G i (5–12). However, pertussis toxin pretreatment did not affect SRIF stimulation of sst2A receptor phosphorylation. These results with the sst2A receptor agree with observations with m2 muscarinic receptor, another inhibitory G protein-coupled receptor known to be phosphorylated by GRKs (50). Hormone stimulation of sst2A receptor phosphorylation by a second messenger-independent mechanism thus suggests the involvement of G protein-coupled receptor kinases, although our experiments do not rule out the possibility that second messenger cascades activated via pertussis toxin-insensitive G proteins mediate SRIF stimulation of sst2A receptor phosphorylation. However, two other observations argue against this possibility. First, even though PIA, carbachol, and SRIF produce the same inhibitory effect on hormone secretion, adenyl cyclase activity, and intracellular calcium in GH cells (41, 51) neither PIA nor carbachol increased sst2A receptor phosphorylation. Thus activation of adenosine or muscarinic receptors does not produce the same effect as does occupancy of the sst2A receptor with agonist. Second, stimulation of sst2A receptor phosphorylation occurred at relatively high concentrations of hormone. By analogy to the β-adrenergic receptor, where much lower agonist concentrations are required to stimulate receptor phosphorylation by cAMP-dependent protein kinase than by GRKs (30), the necessity for high SRIF concentrations for sst2A receptor phosphorylation suggests that the agonist-occupied receptor is being preferentially phosphorylated by GRKs. Indeed, Mayor et al. (52) reported that homologous desensitization of S49 mouse lymphoma cells with SRIF occurred concurrently with the translocation of a G protein-coupled receptor kinase from the cytoplasm to the plasma membrane. While these results are highly suggestive, additional experiments will be necessary to directly demonstrate a role for G protein-coupled receptor kinases in sst2A receptor phosphorylation.

Interestingly, pharmacological activation of several second messenger-regulated kinases showed that phosphorylation of the sst2A receptor was specifically stimulated following a 15-min incubation with the protein kinase C activator, phorbol 12-myristate 13-acetate (Fig. 8). Although heterologous activation of protein kinase C can regulate sst2A receptor function, it is unlikely that this kinase catalyzed the SRIF-stimulated phosphorylation because incubation with SRIF did not increase phospholipase C activity in GH-R2 cells as assayed by inositol triphosphate accumulation. Moreover, in preliminary studies we found that the amount of 32P incorporation into the sst2A receptor following incubation with maximal concentrations of both SRIF (100 nM) and PMA (200 nM) was close to the sum of the incorporation achieved with either agent alone. This observation further argues against the involvement of protein kinase C in ligand-stimulated receptor phosphorylation. Although it is quite likely that the sst2A receptor is a substrate for protein kinase C because the receptor protein contains consensus sequences for protein kinase C phosphorylation (53), it is also possible that protein kinase C indirectly influences the phosphorylation state of the receptor by activating another kinase and/or by inactivating a protein phosphatase.

The functional consequences of protein kinase C-stimulated receptor phosphorylation are unknown. Previous studies showed that a 2–4-h exposure of pancreatic acinar cells (54) or GH3 cells (55) to phorbol ester decreased SRIF binding, with no change in binding affinity observed in the acinar cells (54). Incubation with phorbol ester also attenuated SRIF inhibition of adenyl cyclase activity in GH3 cells (26). However, from these early studies one cannot assess the direct impact, if any, of protein kinase C phosphorylation on the sst2A receptor. Our observation that protein kinase C activation leads to a dramatic increase in sst2A receptor phosphorylation paves the way for the critical analysis of the mechanism by which this phosphorylation occurs as well as its biological consequences.

Although the sst2A receptor also contains putative consensus sequences for protein kinase A phosphorylation (53), stimulation of cAMP synthesis with forskolin did not increase 32P incorporation into the sst2A receptor (Fig. 8). The ineffectiveness of forskolin in stimulating sst2A receptor phosphorylation raised the possibility that phosphorylation was not detected because of a potential experimental problem. As was the case for the bombesin receptor (37), phosphorylation of residues within the region of the sst2A receptor recognized by our antibody could inhibit receptor immunoprecipitation. Therefore, it was prudent to assess the ability of the antibody to recognize the receptor both before and after kinase activation. As shown in Fig. 9, neither SRIF, PMA, nor forskolin altered sst2A receptor immunoprecipitation efficiency. Thus, the signal intensity of phosphorylated sst2A receptor in these studies accurately reflects the incorporation of 32P into the purified receptor.

Agonist- and PMA-stimulated sst2A receptor phosphorylations occur at multiple sites as 32P labeling of phosphoserine, phosphothreonine, and phosphotyrosine residues was increased (Fig. 10). However, phosphoserine was the predominantly labeled residue under all hydrolysis conditions tested, suggesting that the sst2A receptor is phosphorylated primarily...
on serine(s). Identification of these phosphorylation sites will allow a critical examination of the causal relationship between sst2A receptor phosphorylation, desensitization, and internalization.

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