Agonist-induced Phosphorylation of Somatostatin Receptor Subtype 1 (Sst1)

RELATIONSHIP TO DESENSITIZATION AND INTERNALIZATION*

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The sst1 somatostatin (SRIF) receptor subtype is widely expressed in the endocrine, gastrointestinal, and neuronal systems as well as in hormone-sensitive tumors, yet little is known about its regulation. Here we investigated the desensitization, internalization, and phosphorylation of sst1 expressed in CHO-K1 cells. Treatment of cells with 100 nM SRIF for 30 min reduced maximal SRIF inhibition of adenylyl cyclase from 40 to 10%. This desensitization was rapid (t1/2 < 2 min) and dependent on agonist concentration (EC50 = 2 nm). However, internalization of receptor-bound ligand occurred slowly (t1/2 > 180 min). Incubation of cells with SRIF also caused a rapid (t1/2 < 2 min) increase in sst1 receptor phosphorylation in a dose-dependent manner (EC50 = 1.3 nm), as determined in a mobility shift phosphorylation assay. Receptor phosphorylation was not affected by pertussis toxin, indicating a requirement for receptor occupancy rather than signaling. The protein kinase C activator, phorbol 12-myristate 13-acetate also stimulated sst1 receptor phosphorylation whereas forskolin did not. Both agonist- and phorbol 12-myristate 13-acetate-stimulated receptor phosphorylation occurred mainly on serine. These studies are the first to demonstrate phosphorylation of the sst1 receptor and suggest that phosphorylation mediated uncoupling, rather than sequestration, leads to its desensitization.

The two physiologically active somatostatin (SRIF)1 peptides, SRIF14 and SRIF28, potently regulate numerous endocrine, exocrine, and neuronal functions by interacting with a family of six G protein-coupled receptors (sst1, sst2A, sst2B, sst3, sst4, and sst5) (1–4). The cellular changes induced by sst receptors include inhibition of secretion, modulation of neuronal transmission, and smooth muscle contraction, as well as inhibition of proliferation and stimulation of apoptosis. The sst1 receptor subtype is particularly widely distributed in the gastrointestinal tract (5, 6) and the brain (7, 8) as well as being expressed in several other normal tissues (9, 10). Additionally, sst1 is found in neuroendocrine, prostate, and mammary tumors (11–13). Thus, this receptor subtype is believed to mediate many of the central and peripheral actions of SRIF as well as to present a potential target for cancer therapy and diagnosis (1–4, 14).

The sst1 receptor, like other members of the somatostatin receptor family, is now known to inhibit adenylyl cyclase by interacting with pertussis toxin-sensitive G/G_i proteins (15–17). Other signaling mechanisms potently regulated by the sst1 receptor in a pertussis toxin-sensitive manner include inhibition of Ca2+ influx, membrane hyperpolarization, activation of protein tyrosine phosphatases, and stimulation of the mitogen-activated protein kinase cascade (18–20). However, the sst1 receptor has also been shown to act via pertussis toxin-insensitive mechanisms. Both activation of Na+/H+ exchange and potentiation of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid/kainate current responses to glutamate are unaffected by pertussis toxin pretreatment (21, 22). Thus, sst1 appears capable of signaling via several classes of G proteins.

Exposure of G protein-coupled receptors to agonist usually leads desensitization of receptor responsiveness as well as to initiation of intracellular signaling (23–25). Such receptor desensitization can be produced by an uncoupling of the receptor from G proteins, by receptor down-regulation or, most often, by a combination of the two mechanisms. The molecular events leading to uncoupling are thought to involve increased phosphorylation of the agonist-activated receptor followed by the binding of the phosphorylated receptor to arrestins, which then block further receptor-G protein interactions. Receptor down-regulation usually results from an increased rate of receptor internalization following agonist occupancy and this process is also thought to be triggered by receptor phosphorylation and arrestin binding because of the observation that arrestin can act as an adaptor to link G protein-coupled receptors to clathrin-mediated endocytosis (23–25). Recently, the sst2A and sst3 receptors were shown to be rapidly phosphorylated following SRIF treatment (26–28). However, little is known about the molecular mechanisms responsible for the regulation of the sst1 receptor subtype. Thus, the objective of the present study was to examine the role of sst1 receptor phosphorylation, uncoupling and internalization in sst1 receptor desensitization.

EXPERIMENTAL PROCEDURES

Hormones and Supplies—Cell culture medium and G418 were purchased from Life Technologies, Inc. (Grand Island, NY). The sst1 receptor antiserum (R1–201) has been shown to specifically recognize only this sst receptor subtype (17). Leupeptin, phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, bacitracin, Nonidet P-40, pertussis toxin, bacterial alkaline phosphatase, and Protein A were obtained from Sigma. N-Dodecyl-β-D-maltoside was purchased from Calbiochem (La Jolla, CA). CNBr-activated Sepharose 4B was from Amersham Phar-

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1 The abbreviations used are: SRIF, somatostatin; sst, somatostatin receptor subtype; FMA, phorbol 12-myristate 13-acetate; TACT, N,N’,N”-triacetychitotriose; WGA, wheat germ agglutinin; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; PKC, protein kinase C; ER, endoplasmic reticulum; Endo H, endo-N-acetylgalactosaminidase H.
macia Biotech (Uppsala, Sweden). Bradford reagent and reagents for electrophoresis and Western blotting were obtained from Bio-Rad. Carrier-free Na\(^{221}\) was from Amersham Pharmacia Biotech. \([\alpha-\beta^32P]ATP\) was obtained from PerkinElmer Life Sciences. Phosphate-free Dulbecco’s modified Eagle’s medium and \([\beta^32P]\)orthophosphate were purchased from United Biomedical (Costa Mesa, CA). All other reagents were of the best grade available and purchased from common suppliers.

**Cell Culture**—The cloned CHO-R1 cell line was generated by stable transfection of CHO-K1 cells with the rat sst1 receptor followed by clonal selection (15). The cell line contains 98% ± 90% (n = 4)fold of receptor/mg of membrane protein with an affinity \((K_d)\) of \(240 ± 100 \mu \text{M}\) for \([\text{Tyr}^{11}]\)SRIF. Cells were grown in F12 medium containing 10% fetal calf serum and 250 µg/ml G418. Experimental cultures were plated in medium without G418 and used 3–4 days later with a medium change 18–24 h prior to use. Experiments were carried out with cells plated in 100-mm dishes except for whole cell binding experiments, for which 35-mm wells were used.

**Membrane Preparation**—Cells were pretreated in a CO\(_2\) incubator at 37 °C with 100 nM SRIF or carrier in serum-free F12 medium containing 5 mg/ml lactalbumin hydrolysate and 20 mM HEPES, pH 7.4 (F12LH). The 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, 0.1 mg/ml bovine serum albumin). Washed cells were scraped into HME plus 10 µg/ml leupeptin, 20 µg/ml tetrasodium pyrophosphate, and 0.1 µM okadaic acid and homogenized with a Dounce homogenizer. The homogenates were centrifuged on a step gradient of 23 and 43% sucrose in HE buffer (20 mM Hepes, pH 8.0, 1 mM EDTA) and the fraction at the 23:43% interface was collected and stored at −80 °C (29).

**Adenylyl Cyclase Assay**—Membranes (5–10 µg of protein/tube) were assayed in triplicate for adenylyl cyclase activity at 30 °C for 10 min in the presence of 4 mM added MgCl\(_2\) (29).

**Radioligand Binding and Internalization—**[\text{Tyr}^{11}]SRIF was radioiodinated using chloramine T and subsequently purified by reverse-phase high performance liquid chromatography as described previously (17). CHO-R1 cells were incubated either at 37 or 4 °C in F12LH containing ~100,000 cpm of [\text{Tyr}^{11}]SRIF in the absence or presence of 100 nM unlabeled SRIF (30). Following 4 °C binding incubations, cells were rinsed free of unbound trace and subsequently incubated in fresh 37 °C buffer to allow internalization of the receptor-bound ligand. To determine the distribution of bound radioligand, cells were incubated on ice for 5 min in acetic acid-buffered saline (200 mM acetic acid, 500 mM NaCl, pH 2.5). After collection of the acidic buffer the cells were dissolved in 0.1 N NaOH. The radioactivity in both the acid wash, representing surface-bound ligand, and in cell pellets representing receptor internalization ligand, was measured (30). 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).

**Phosphorylation and Deglycosylation of \([32P]\)-Labeled Receptor—**Metabolic labeling of cells with \([32P]\)PO\(_4\) and subsequent immunoprecipitation of the receptor carried out as described previously for the sst1 receptor (26). Briefly, cells were incubated for 3 h in phosphate-free Dulbecco’s modified Eagle’s medium containing 1 mM of \([\beta^32P]\)orthophosphate. Hormones and pharmacological agents were then added directly to the labeling medium, and the cells were further incubated at 37 °C under 5% CO\(_2\) for the indicated times. The cells were then scraped into cold Hepes-buffered saline with protease and phosphatase inhibitors (HBS: 150 mM NaCl, 20 mM Hepes, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml soybean trypsin inhibitor, 10 µg/ml leupeptin, 50 µg/ml bacitracin, 5 mM EDTA, 3 mM EGTA, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 0.1 mM orthovanadate, 100 mM okadaic acid). Following centrifugation, the cell pellet was solubilized in lysis buffer (HBS containing 4 mg/ml dodecyl-β-D-maltoside, 0.1 SDS%) and homogenized with a Dounce homogenizer. The homogenates were centrifuged (100,000 g) for 90 min followed by incubation at 4 °C for 90 min. Following centrifugation, the WGA-agarose was washed vigorously with lysis buffer. In some experiments adsorbed glycoproteins were then eluted at 37 °C for 30 min with lysis buffer containing 3 mM \(N^\gamma,N^\delta,N^\epsilon\)-triacetylatedtiristione (TACT) (Sigma) and 0.5% SDS (v/v). In most experiments, however, adsorbed glycoproteins were deglycosylated by incubating the washed WGA-agarose at 37 °C overnight with lysis buffer (pH 7.4) containing 0.1% SDS (v/v) and 10 units/ml of peptide-N-glycosidase F (PNGase F, Roche Molecular Biochemicals, Indianapolis, IN) (32). This enzyme, which catalyzes the cleavage of \(N\)-glycosidically linked carbohydrate chains between \(N\)-acetylglucosamine and asparagine, caused the release of most of the receptor from the WGA-agarose. After either the TACT or glycosidase treatment, the supernatant containing the dissociated receptor was incubated with a 1:200 dilution of the anti-sst1 receptor antibody R1–201 (17) at 4 °C for ≥90 min followed by incubation at 4 °C for 60 min with 25 µl (packed volume) of protein A-Sepharose 4B. Following centrifugation, the Sepharose beads were washed as described previously (26) and the immunoprecipitated proteins were solubilized in sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% 2-mercaptoethanol (v/v), 6 M urea, 20% glycerol, pH 6.8) at 37 °C for 60 min, and resolved on 12% SDS-polyacrylamide gels.

**Purification and Deglycosylation of Unlabeled Receptor and Detection by Immunoblotting—**CHO-R1 cells were incubated in growth medium in the presence or absence of SRIF, PMA, or forskolin at 37 °C for the times indicated. The cells were then scraped into cold Hepes-buffered saline, pelleted, and solubilized in lysis buffer 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 supernatant was assayed by the method of Bradford (31). Following adsorption to WGA-agarose and deglycosylation by PNGase F as described above, the supernatant was incubated for ≥90 min at 4 °C with anti-sst1 receptor IgG covalently coupled to Protein A-Sepharose 4B. The immunoprecipitated proteins were dissolved in sample buffer without reducing reagent for 60 min at 37 °C. After removal of the Sepharose beads and addition of 10% 2-mercaptoethanol (v/v) proteins were resolved on 12% SDS-polyacrylamide gels.

To investigate the subcellular distribution of phosphorylated receptors, sst1 was also deglycosylated with endo-\(N\)-acetylglucosaminidase H (Endo H, Roche Molecular Biochemicals), an enzyme which hydrolyzes high-mannose oligosaccharides from glycoproteins (33). Following incubation with WGA-agarose as described above, the washed beads were incubated with lysis buffer (pH 5.8) containing 0.02% SDS (v/v) and 75 milliunits/ml of Endo H at 37 °C overnight. Replica aliquots were incubated with PNGase F at the same time. Following deglycosylation, the WGA-agarose beads were centrifuged and proteins released into the supernatant were precipitated at 4 °C for 2 h by 12.5% (v/v) trichloroacetic acid. Both the trichloroacetic acid-precipitated proteins and the proteins remaining adsorbed to the WGA beads were solubilized in sample buffer and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described above.

Resolved proteins were transferred to PVDF membrane and immunoblotted with 1:5000 dilution of anti-sst1 antibody R1–201 as described previously (13). Immunoreactive proteins were detected with an ECL detection system (Amersham Pharmacia Biotech).

For phosphatase treatment, anti-receptor IgG-Sepharose beads with adsorbed receptor were washed once with phosphatase buffer (20 mM Hepes, pH 8.0, 25 mM KCl, 15 mM MgCl\(_2\)) and then incubated with 100 µl of buffer containing 4 mg/ml dodecyl-β-D-maltoside, 0.1 SDS%, and 5 units of bacterial alkaline phosphatase (Sigma) at 37 °C for 1 h (34). After centrifugation, the immunoprecipitated receptor was eluted and analyzed as described above.

**Phosphoamino Acid Analysis—**Following phosphorylation and purification of the sst1 receptor as described above, SDS-PAGE-resolved proteins were transferred to PVDF membrane, and the \([32P]\)PO\(_4\)-containing bands were localized by autoradiography. Membrane pieces containing selected bands were excised and incubated in 50 µl of 5.7 N HCl (Pierce) at 110 °C for 30 min (26). Phosphoamino acids were resolved by two-dimensional thin layer electrophoresis on cellulose plates (26).

**Methods—**Protein A-Sepharose 4B was covalently coupled to CNBr-activated Sepharose 4B according to the manufacturer’s instructions (Amersham Pharmacia Biotech). Anti-receptor IgG was covalently coupled to protein A-Sepharose as described previously (17). Fitted values for the maximal effect and the EC\(_{50}\) were obtained by least squares nonlinear regression analysis of dose-response curves using the program D/R (Biomedical Computer, Inc. Houston, TX). \([32P]\)PO\(_4\)-Labeled bands were quantitated using a PhosphorImager and visualized by autoradiography on Biomax MS film (Kodak, Rochester, NY). Unless otherwise indicated results of a representative experiment are shown. All experiments were repeated at least 2 times.
**RESULTS**

Desensitization of the Sst1 Receptor—To investigate the susceptibility of the rat sst1 receptor to desensitization, CHO-R1 cells were incubated in the absence or presence of 100 nM SRIF for 30 min at 37 °C and the effect of pretreatment on hormonal regulation of membrane adenylyl cyclase activity was determined (Fig. 1). SRIF pretreatment did not affect either basal or forskolin-stimulated adenylyl cyclase activity. Cyclase activity was 6.0 ± 1.5 pmol/min/mg (mean ± S.E., n = 3) under basal conditions and 263 ± 43 pmol/min/mg (n = 5) in the presence of 10 μM forskolin. In membranes from untreated cells SRIF inhibited forskolin-stimulated adenylyl cyclase activity with an EC_{50} of 1.62 ± 0.75 nM (n = 5) (Fig. 1). Maximal inhibition was 42.0 ± 7.4% (n = 5). Preincubation of cells with 100 nM SRIF markedly attenuated the efficacy of SRIF inhibition (maximal inhibition = 10.6 ± 2.2%, n = 5). Because cyclase inhibition was so small in pretreated cells, it was not possible to calculate the EC_{50} for SRIF.

To determine the time dependence of sst1 desensitization, cells were incubated in the absence or presence of 100 nM SRIF for varying times and the effect of the different pretreatments on membrane adenylyl cyclase activity was subsequently measured (Fig. 2). The sst1 receptor was maximally desensitized within 2 min and desensitization was maintained for at least 30 min.

The concentration dependence for sst1 desensitization was analyzed by incubating cells for 5 min in the presence of varying amounts of SRIF and then measuring the effect on membrane adenylyl cyclase activity. Both the potency and the efficacy of SRIF inhibition of adenylyl cyclase were diminished by pretreatment with low concentrations of SRIF. The EC_{50} for SRIF inhibition of adenylyl cyclase was increased from 2.4 ± 1.0 nM in control membranes to 6.9 ± 2.5 nM after pretreatment with 1 nM SRIF and the maximal inhibition was reduced from 42.6 ± 2.7 to 30.3 ± 2.4% (Fig. 3, top panel). Desensitization was further increased at higher peptide doses and pretreatment with 100 nM SRIF reduced maximal cyclase inhibition to 8.3 ± 2.4% (Fig. 3, top panel). The EC_{50} for SRIF-induced desensitization was 2 nM (Fig. 3, bottom panel). Together, these studies demonstrate that exposure to agonist results in homologous desensitization of the sst1 receptor in a time- and concentration-dependent manner.

Internalization of the Sst1 Receptor—To ascertain whether agonist binding induced the internalization of the ligand–receptor complex, cells were incubated for 4 h at 4 °C with [125I]-Tyr^1]SRIF to occupy cell surface receptors, washed to remove unbound peptide, and then warmed to 37 °C for varying times (Fig. 4, top panel). Internalization of the receptor-bound ligand occurred slowly and only 30% of the complex was internalized even after a 60-min incubation at 37 °C. The rate of internalization was fit to a first-order rate equation and the half-time for endocytosis of the receptor-ligand complex was calculated to be 180 min.

Consistent with the slow rate of internalization of the sst1 receptor-ligand complex observed in temperature-jump experiments (Fig. 4, top panel), analysis of the distribution of receptor-bound ligand during continuous incubation at 37 °C showed that only 15% of the bound ligand was present intracellularly at steady state (Fig. 4, bottom panel).

Agonist-stimulated Phosphorylation of the Sst1 Receptor—We previously showed that the R1–201 antibody specifically recognizes the sst1 receptor (17). Furthermore, photoaffinity labeling and immunoprecipitation experiments using CHO-R1 cells demonstrated that the sst1 receptor migrates as a broad 60-kDa band on SDS-PAGE (17). We next wanted to determine whether SRIF was able to stimulate the incorporation of [32P]PO_4 into the sst1 receptor protein. To ensure that the R1–201 antibody was equally capable of recognizing the sst1 receptor before and after SRIF stimulation, CHO-R1 cells were incubated with or without 100 nM SRIF for 15 min and the solubilized receptors were subsequently immunoprecipitated with antibody covalently coupled to Sepharose. Analysis of the immunoprecipitated proteins by SDS-PAGE and immunoblotting with receptor antibody showed that SRIF treatment did not alter the immunoprecipitation efficiency of sst1 (data not shown).
To directly assess agonist-stimulated receptor phosphorylation, CHO-R1 cells were labeled with \[^{32}\text{P} \]orthophosphate and incubated in the absence or presence of 100 nM SRIF for 15 min. After detergent solubilization, the receptor was purified by lectin affinity chromatography followed by immunoprecipitation with receptor antiserum and then analyzed by SDS-PAGE and autoradiography. A low level of basal phosphorylation was observed in the 60-kDa broad receptor band as well as in a narrower 45-kDa band (Fig. 5, left panel). The 45-kDa band probably represents a nonspecific contaminant because its intensity relative to the 60-kDa band varied markedly between experiments and because SRIF treatment did not affect \[^{32}\text{P} \]incorporation into the 45-kDa protein. In contrast, treatment of cells with 100 nM SRIF increased the amount of \[^{32}\text{P} \]incorporated the 60-kDa sst1 receptor protein 2.2 \(\pm\) 0.4-fold over basal (\(n = 5\)). Therefore, exposure to SRIF increases phosphorylation of the previously identified 60-kDa sst1 receptor protein within 15 min.

We next wanted to determine the time course and dose dependence of receptor phosphorylation. Because receptor phosphorylation was difficult to quantitate in the broad 60-kDa band we attempted to sharpen the migration pattern of the receptor on SDS-PAGE by removing the attached carbohydrate. \[^{32}\text{PO}_{4} \]Labeled CHO-R1 cells were incubated in the absence or presence of 100 nM SRIF for 15 min. After detergent solubilization and adsorption to WGA-agarose, the adsorbed receptor was incubated with PNGase F. The proteins released from the WGA-agarose by deglycosylation were then immunoprecipitated with receptor antiserum and analyzed by SDS-PAGE and autoradiography (Fig. 5, right panel). Two \[^{32}\text{P} \]-labeled bands were observed in control cells after receptor deglycosylation, migrating at 34 and 38 kDa. SRIF treatment led to the appearance of a third, strongly phosphorylated, band at 43 kDa (Fig. 5, right panel). None of the deglycosylated \[^{32}\text{P} \]-labeled proteins were immunoprecipitated by receptor antibody in the presence of 1 \(\mu\)M antigen peptide, indicating that they represented different forms of the sst1 receptor (Fig. 5, right panel). In six independent experiments, a 15-min pretreatment with 100 nM SRIF led to a 4.7 \(\pm\) 1.5-fold increase in the phosphorylation of the 43-kDa receptor band, a sufficiently large increase to permit ready quantitation (see below).
As observed in the absence and presence of bacterial alkaline phosphatase protein A-Sepharose. Replicate aliquots were then incubated in the absence or presence of 100 nm SRIF for 15 min. Following detergent solubilization and adsorption to WGA-agarose, the adsorbed proteins were deglycosylated by incubating the beads overnight at 37 °C with 10 units/ml PNGase F. The proteins released from WGA-agarose by deglycosylation were subjected to immunoprecipitation with receptor antiserum (1:200) in the absence or presence of 1 μM agonist peptide (Ag). In both panels, immunoprecipitated proteins were analyzed by SDS-PAGE and autoradiography.

Moreover, alkaline phosphatase digestion collapsed all receptor bands to a single species at 34 kDa (Fig. 6, top panel). These results demonstrate that both the 38- and 43-kDa bands represent phosphorylated forms of the sst1 receptor protein, with the 43-kDa form being specifically increased by SRIF treatment. Furthermore, the R1-201 antibody is able to recognize the sst1 receptor in its different phosphorylated states.

The results in Fig. 6, top panel, show that not all sst1 receptors are phosphorylated after SRIF treatment; a substantial fraction of the receptors from SRIF-treated cells continue to migrate at 34 kDa. We therefore tested whether phosphorylation resistant sst1 receptors consisted of immature, intracellular proteins. Receptors in the ER will contain core, high mannose carbohydrates that are sensitive to digest with the enzyme Endo H. In contrast, receptors that have successfully traveled through the ER and cis-Golgi will possess mature, complex carbohydrates that would be Endo H-resistant. Mature carbohydrates would be expected either if the sst1 receptor was on the cell surface or if it was sequestered in a post-Golgi intracellular compartment such as endosomes.

CHO-R1 cells were incubated in the absence or presence of 100 nm SRIF for 15 min as before. Following detergent solubilization and adsorption to WGA-agarose, the adsorbed proteins were incubated with either PNGase F or Endo H (Fig. 6, bottom panel). Most of the receptor was dissociated from the WGA-agarose by the PNGase F incubation and the same pattern of receptor bands was observed both without and with SRIF treatment as above (Fig. 6, bottom left panel). In contrast, Endo H treatment released only a small fraction of the sst1 receptors from WGA-agarose (Fig. 6, bottom right panel) indicating that a relatively small portion of the receptors are in the ER. However, those sst1 receptors which were deglycosylated by Endo H were not affected by SRIF pretreatment; Endo H-digested receptor from both control cells and from SRIF-treated cells migrated at 34 kDa. Thus, the high mannose containing precursor form of the sst1 receptor in the ER is resistant to SRIF-stimulated phosphorylation, as would be expected from its inaccessibility to extracellular ligand. These results indicate that intracellular compartmentation contributes to the resistance of a portion of the sst1 receptor population to SRIF-stimulated phosphorylation.
Time Course and Dose Dependence of Sst1 Receptor Phosphorylation—If sst1 receptor phosphorylation plays a role in agonist-stimulated desensitization or internalization, phosphorylation should be increased within the time frame of one of these regulatory events. To test this hypothesis, $^{32}$P-labeled CHO-R1 cells were incubated with 100 nM SRIF for various times. Following lectin chromatography and deglycosylation by PNGase F, the sst1 receptor was immunoprecipitated with receptor antiserum and analyzed by SDS-PAGE and autoradiography (Fig. 7). Stimulation of sst1 phosphorylation reached 90% of maximum after a 2-min incubation, was complete by 5 min, and was then maintained for at least 15 min (Fig. 7).

Agonist stimulation of sst1 receptor phosphorylation was also concentration dependent (Fig. 8). During a 15-min incubation, phosphorylation of the sst1 receptor was significantly elevated by 1 nM SRIF and was further increased at higher doses up to a maximal effect with 100 nM peptide (Fig. 8). The EC$_{50}$ for SRIF-stimulated receptor phosphorylation was 1.3 ± 0.5 nM (n = 2). Interestingly, Fig. 8 also shows that the $^{32}$P labeling of the 34-kDa band is reduced at the same time as the incorporation into the 43-kDa band is increased, indicating that increased phosphorylation of the 34-kDa band gives rise to the 43-kDa form of the receptor. In six independent experiments, a 15-min treatment with 100 nM SRIF decreased $^{32}$PO$_4$ incorporation into the 34-kDa receptor band by 36.7 ± 10.5% at the same time that it increased $^{32}$PO$_4$ incorporation into the 43-kDa band (arrow) from two independent experiments.

Mechanisms Involved in Sst1 Receptor Phosphorylation—To assess the importance of sst1 receptor-G$_{i/o}$ coupling for SRIF-induced receptor phosphorylation, CHO-R1 cells were pretreated with 100 ng/ml pertussis toxin for 24 h. This treatment abolished SRIF inhibition of forskolin-stimulated adenyl cyclase activity (data not shown) as described previously (15). However, SRIF-induced phosphorylation of the sst1 receptor was unaffected by pertussis toxin pretreatment (Fig. 9). Therefore, functional interaction of the sst1 receptor with pertussis toxin-sensitive G proteins is not necessary for agonist-induced sst1 receptor phosphorylation.
We further evaluated the effect of second messenger-regulated protein kinases on sst1 receptor phosphorylation. Either \(^{32}\)PO\(_4\)-labeled CHO-R1 cells (Fig. 10, left panels) or unlabeled cells (Fig. 10, right panel) were incubated for 15 min with either no addition (control), or the indicated agents followed by sst1 receptor purification and analysis. Both SRIF and PMA increased \(^{32}\)P incorporation. However, whereas SRIF primarily increased labeling of the 43-kDa receptor band, PMA stimulated \(^{32}\)P incorporation mainly into the 38-kDa band (Fig. 10, left panel): the ratio of \(^{32}\)P in the 43:38-kDa bands was 1.38 in SRIF-treated cells and 0.65 in PMA-treated cells. Comparable results were observed by immunoblotting the sst1 receptor from unlabeled cells (Fig. 10, right panel). In two independent experiments PMA increased \(^{32}\)P incorporation into the combined 38- plus 43-kDa bands 2.63 \(\pm\) 0.08-fold (mean \(\pm\) range). In contrast, forskolin did not affect sst1 receptor phosphorylation (Fig. 10, left panel). Following forskolin treatment \(^{32}\)PO\(_4\) incorporation into the combined 38- plus 43-kDa bands was 1.18 \(\pm\) 0.11 (mean \(\pm\) range, \(n = 2\)) times control.

Since SRIF and PMA both increased sst1 receptor phosphorylation, we next determined whether their effects were additive. Interestingly, simultaneous addition of agonist and PMA produced the same effect as agonist alone (Fig. 10, left bottom panel). The phosphorylation of the 43-kDa band was preferentially increased by the combined treatment with 1.20 \(\pm\) 0.13 times as much \(^{32}\)P incorporated into the 43-kDa band as into the 38-kDa band (\(n = 2\)). Furthermore, \(^{32}\)P incorporation into the combined 38- plus 43-kDa bands was stimulated 2.6-fold in cells treated with both SRIF plus PMA compared with a 2.8-fold stimulation with SRIF alone and a 2.6-fold stimulation with PMA alone.

Together, these results demonstrate that protein kinase A activation does not affect sst1 receptor phosphorylation. In contrast, protein kinase C and SRIF both stimulate the phosphorylation of sst1 leading to the formation of different phosphorylated products which can be distinguished by their electrophoretic mobility. These results suggest that stimulation of sst1 receptor phosphorylation by SRIF and PMA involves different kinases and target different phosphorylation sites. Since combined treatment with SRIF and PMA produced the same effect as SRIF alone, hormone-stimulated phosphorylation appears to prevent the protein kinase C-catalyzed reaction.

**Phosphoamino Acid Analysis of Phosphorylated Sst1**—To identify the phosphorylated residues in the sst1 receptor, phosphoamino acid analysis was carried out with receptor from cells incubated with either 100 nM SRIF or 200 nM PMA for 15 min. The 43-kDa form of the receptor produced by SRIF treatment and the combined 43- and 38-kDa forms produced by PMA treatment were subjected to acid hydrolysis for 30 min and analyzed for the presence of different phosphoamino acids (Fig. 11). With both treatments, the most heavily labeled residue was phosphoserine, although a small amount of radioactivity was also incorporated into phosphothreonine. Therefore, SRIF- and PMA-stimulated sst1 receptor phosphorylation occurs primarily on serine residues and secondarily on threonine.

**DISCUSSION**

We show for the first time that the sst1 somatostatin receptor undergoes rapid, agonist-stimulated phosphorylation and that receptor phosphorylation correlates with homologous desensitization but not with receptor internalization. Our results indicate that receptor uncoupling rather than receptor endocytosis is responsible for sst1 desensitization and suggest that receptor phosphorylation is involved in the desensitization process.
Previous studies had suggested that the internalization of the sst1 receptor was species specific. Thus, hormone treatment produced endocytosis of the rat sst1 receptor into intracellular vesicles within minutes (35). In contrast, somatostatin did not stimulate the internalization of human sst1 (36, 37). These results were surprising in view of the few differences between the rat and human sst1 receptor sequences. In fact, the intracellular domains of the rat (X61630) and human (M81829) sst1 differ by only two amino acids: at the COOH terminus the rat sst1 receptor contains the sequence ASRISTL whereas the human receptor contains the sequence TSRRITTL. Our observation (Fig. 4) that the rat sst1 receptor internalized as slowly in CHO-K1 cells as did the human receptor (36) shows that the small sequence divergence between these species does not significantly affect the rate of receptor internalization when measured in the same host. The study of rat sst1 by Roosterman et al. (35) differed experimentally from ours in two ways. First, we used different parental cells for expression of rat sst1: Roosterman et al. (35) transfected RIN1046-38 cells, a differentiated insulinoma cell line, whereas we used CHO-K1 cells, an established model for studies of G protein-coupled receptor regulation. Second, we studied the native receptor whereas Roosterman et al. (35) used a receptor containing an epitope tag on the C terminus (rat sst1-tag). At present, we do not know whether the observed differences in the rate and extent of hormone-induced internalization of rat sst1 in the two studies result from differences in the internalization machinery between the two host cells or from the presence of an epitope tag on the receptor used by Roosterman et al. (35).

Perhaps it is pertinent in this regard that the endogenous, but unidentified, SRIF receptors in another rat insulinoma cell line, RINm5F cells, were not rapidly internalized following hormone binding (38). These studies emphasize that it will be important to examine receptor processing in cells expressing sst1 endogenously.

Pretreatment of cells with SRIF leads to desensitization of both the rat and human sst1 receptor (35, 39). In HEK cells expressing rat sst1-tag no effect was observed after a 10-min pretreatment with 1 μM SRIF but significant desensitization occurred after preincubation for 30 and 120 min (35). However, the relationship between desensitization and receptor internalization was not examined in this cell line (35). In another study, the human sst1 receptor expressed in CHO-K1 cells was shown to desensitize after treatment with SRIF for 60 min when little receptor internalization had occurred (36, 39). We show here that desensitization of the native rat sst1 receptor proceeds extremely rapidly in CHO-K1 cells, with a half-time less than 2 min. This is well before significant receptor internalization occurs. Thus, desensitization of sst1 results from receptor uncoupling rather than receptor sequestration. This contrasts with the behavior of the sst2A receptor, which undergoes desensitization and internalization at similar rates (26).

The mechanisms mediating sst1 receptor desensitization are not known but, as has been postulated for other G protein-coupled receptors (23–25), may involve receptor phosphorylation. We show for the first time that hormone binding stimulates the phosphorylation of the sst1 receptor. Moreover, we demonstrate that (a) agonist stimulation of sst1 receptor phosphorylation occurs concurrently with receptor desensitization, and (b) the dose-response for SRIF-stimulated receptor phosphorylation and SRIF-induced receptor desensitization are indistinguishable. By analogy to results with other members of the G protein-coupled receptor family, these observations indicate that receptor phosphorylation is important in sst1 desensitization.

Our first evidence that hormone binding led to phosphorylation of the sst1 receptor was based on increased incorporation of $^{32}$P into immunoprecipitated receptor protein (Fig. 5). However, in subsequent studies we observed that phosphorylation correlated with a decrease in the mobility of the sst1 receptor on SDS-PAGE, and that this change in mobility was reversed by alkaline phosphatase digestion, as would be expected for a phosphorylation dependent effect. Although such mobility shifts are often exploited in studies of kinase signaling cascades, they have been used rarely in the study of G protein-coupled receptor phosphorylation (40, 41). The ability to distinguish the phosphorylated receptor from the unphosphorylated form on Western blots enabled us to determine the fraction of receptors phosphorylated. Given the close correlation between receptor phosphorylation and desensitization, we were surprised to find that less than half of the sst1 receptors were phosphorylated following agonist stimulation. Since this partial phosphorylation was associated with essentially complete desensitization, we surmised that the unphosphorylated receptors were present in an inactive receptor pool, i.e. not coupled to cyclase inhibition.

A previous report showing that a substantial portion of sst1 receptors are localized intracellularly in unstimulated CHO cells (39) suggested that the fraction of receptors resistant to phosphorylation may derive from this cytoplasmic pool. To determine whether the phosphorylated and phosphorylation resistant forms of sst1 were present in different intracellular compartments, we exploited the specificity of the carbohydrate selective endoglycosidase, Endo H. This enzyme can differentiate between immature receptors in the ER which contain high mannose carbohydrates and are sensitive to Endo H digestion and fully processed receptors containing complex N-linked oligosaccharides which are Endo H resistant (42). Our observation that Endo H-sensitive receptors were not phosphorylated after SRIF exposure demonstrated that only fully processed, mature receptors were substrates for hormone-stimulated phosphorylation. Thus, different sst1 receptor pools were differentially sensitive to agonist-stimulated phosphorylation. Unfortunately, because fully processed endosomal receptor pools cannot be distinguished from plasma membrane receptors by endoglycosidase digestion we do not yet know whether plasma membrane sst1 represents the receptor population which is susceptible to hormone-stimulated phosphorylation. Nonetheless, our results clearly show that phosphorylation of a receptor subpopulation is sufficient to cause complete desensitization of SRIF inhibition of adenylyl cyclase. If this is true for other G protein-coupled receptors, it may help explain why receptor phosphorylation has been so difficult to detect in some instances. Our results suggest that the development of a mobility shift assay with deglycosylated receptors may help overcome such sensitivity problems.

Two types of kinases are known to phosphorylate G protein-coupled receptors: second messenger-activated kinases and G protein-coupled receptor kinases. The latter preferentially phosphorylate agonist-occupied, activated receptors and are independent of second messenger formation. Forskolin did not affect sst1 receptor phosphorylation despite the presence of protein kinase A consensus sites in the intracellular regions of the receptor (43). However, both agonist and the protein kinase C activator, PMA, stimulated sst1 receptor phosphorylation. Two observations indicated that these phosphorylation reactions are catalyzed by different kinases. First, the mobility of the phosphorylated sst1 receptor is different following SRIF and PMA stimulation: SRIF increases $^{32}$P incorporation into a 43-kDa band whereas PMA primarily stimulates labeling of a 38-kDa band (Fig. 10). This difference in mobility must result
from phosphorylation of the receptor at different sites, since it disappears after alkaline phosphatase treatment, and therefore indicates that SRIF and PMA stimulate kinases with different substrate specificities. Second, SRIF-stimulated phosphorylation does not require signal transduction. Although sst1 activation can weakly stimulate PIP2 hydrolysis in CHO-K1 cells and thereby could potentially increase PKC activity, this effect is prevented by pertussis toxin treatment (44). However, since pertussis toxin did not affect SRIF-induced sst1 phosphorylation, PKC cannot be involved in the stimulation of receptor phosphorylation by agonist. Based on these results, we conclude that the sst1 phosphorylation produced by SRIF and PMA must involve different kinases and must generate different products. Interestingly, simultaneous treatment with both SRIF and PMA produced the same effect as SRIF alone, indicating that agonist may prevent receptor phosphorylation by heterologous kinases.

The inability of pertussis toxin to block SRIF-stimulated sst1 phosphorylation also suggests that agonist-stimulated phosphorylation is independent of receptor-G protein coupling, a characteristic of G protein-coupled receptor kinase-catalyzed receptor phosphorylation. Although it remains possible that a second messenger cascade which is activated by pertussis toxin-insensitive G proteins mediates SRIF stimulation of sst1 receptor phosphorylation, this seems unlikely for two reasons. First, agonist binding to the sst1 receptor is completely resistant to GTP inhibition after pertussis toxin treatment of CHO-K1 cells (17). Second, all sst1-mediated signaling in CHO-K1 cells, including cyclase inhibition, inositol 1,4,5-trisphosphate formation, tyrosine phosphatase stimulation, and mitogen-activated protein kinase activation are blocked by pertussis toxin (15, 20, 44). Together, these observations provide strong support for the conclusion that the sst1 receptor is coupled exclusively to pertussis toxin-sensitive G proteins in this cell line. Therefore, the inability of pertussis toxin to affect SRIF-stimulated sst1 receptor phosphorylation demonstrates that this phosphorylation is independent of receptor-G protein coupling and rules out the involvement of second messenger-activated kinases.

As described here for the sst1 receptor, we had previously shown that the sst2A receptor subtype is also phosphorylated independently by PMA and agonist stimulation but is unaffected by protein kinase A activation (26, 27). Recent studies demonstrated that PKC stimulation increases the rate of sst2A receptor internalization (27). In contrast, PMA did not induce or inhibit the endocytosis of the rat sst1-tag receptor in RIN1046-38 (35). Thus, the biological significance of the PKC-mediated sst1 receptor phosphorylation remains to be elucidated.

As a first step toward identifying the sites of sst1 receptor phosphorylation, we determined which amino acids were phosphorylated following agonist and PMA stimulation. Although a small amount of labeled phosphothreonine was observed, phosphoserine was the predominantly labeled residue found under both conditions. Clearly identification of the sites phosphorylated upon hormone and PMA stimulation will be crucial for determining the functional consequences of sst1 receptor phosphorylation and for elucidating the relationship between receptor phosphorylation and desensitization.

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