The sst2A receptor is expressed in the endocrine, gastrointestinal, and neuronal systems as well as in many hormone-sensitive tumors. This receptor is rapidly internalized and phosphorylated in growth hormone-R2 pituitary cells following somatostatin binding (Hipkin, R. W., Friedman, J., Clark, R. B., Eppler, C. M., and Schonbrunn, A. (1997) J. Biol. Chem. 272, 13869–13876). The protein kinase C (PKC) activator, phorbol 12-myristate 13-acetate (PMA), also stimulates sst2A phosphorylation. Here we examine the mechanisms and consequences of PKA and agonist-induced sst2A phosphorylation. Like somatostatin, both PMA and bombesin increased sst2A receptor phosphorylation within 2 min. The PKC inhibitor GF109203X blocked PMA- and bombesin-stimulated sst2A phosphorylation, whereas stimulation by the somatostatin analog SMS 201–995 was unaffected. Agonist and PMA each stimulated phosphorylation in two receptor domains, the third intracellular loop and the C-terminal tail. Functionally, PMA dramatically increased the internalization of the sst2A receptor-ligand complex. This PMA stimulation was blocked by GF109203X, whereas basal internalization was unaffected. However, neither basal nor PMA-stimulated internalization was altered by pertussis toxin, whereas both were blocked by hypertonic sucrose. Therefore PKC activation and agonist binding stimulate sst2A phosphorylation by distinct mechanisms, and PKC potentiates internalization of the sst2A receptor via clathrin-coated pits. Thus, hormonal stimulation of PKC-coupled receptors may provide a mechanism for regulating the inhibitory actions of somatostatin in target tissue.

For most G protein-coupled receptors, exposure to an agonist leads to a decrease in receptor responsiveness (homologous desensitization) often coincident with internalization of surface receptors (for reviews see Refs. 1 and 2). Additionally, agonist-independent or heterologous desensitization may occur when hormonal activation of one receptor reduces cellular responsiveness through a different receptor system (3). Whereas homologous desensitization may be mediated by either G protein-coupled receptor kinases (GRKs) or second messenger-dependent protein kinases, heterologous desensitization is thought to involve only the latter. GRKs preferentially phosphorylate the agonist-occupied receptor, increasing its affinity for cytoplasmic arrestins, which disrupt receptor G protein coupling and may also act as adaptors for receptor internalization via clathrin-coated pits. In contrast, heterologous desensitization can involve phosphorylation of unoccupied as well as agonist-occupied receptors and may or may not be associated with increased receptor internalization.

The somatostatin peptides (SRIF-14 and SRIF-28) regulate endocrine, exocrine, immune, and neuronal function through binding to a family of six G protein-coupled receptors (sst1, sst2A, sst2B, sst3, sst4, and sst5) (4, 5). Expression of the SRIF receptor 2A subtype (sst2A) in the central nervous system (6), the pituitary (7), the endocrine and exocrine pancreas (8, 9), and the gastrointestinal tract (10) as well as in a variety of neoplasms (11, 12) supports the contention that this receptor isotype mediates many of the physiological and pathological actions of SRIF. Thus, elucidation of the mechanisms involved in sst2A receptor regulation has important implications in understanding SRIF function.

We previously showed that the sst2A receptor is rapidly desensitized, internalized, and phosphorylated following agonist stimulation in GH-R2 cells, a pituitary cell line transfected to express high levels of this receptor subtype (13). Moreover, incubation with the protein kinase C activator, phorbol 12-myristate 13-acetate (PMA) also produced a dramatic increase in receptor phosphorylation (13). Although the signal transduction pathways most potently and widely affected by the sst2A receptor include inhibition of adenylyl cyclase and Ca2+ channels and stimulation of K+ channels (4, 5), recent studies have shown sst2A stimulation of phosphoinositide hydrolysis in transfected COS-7 (14) and F344 pituitary cells (15). Further, SRIF has been shown to increase inositol phosphate levels in several tissues by activating endogenous SRIF receptors (16, 17). Our observation that PMA treatment stimulated sst2A receptor phosphorylation within minutes led us to investigate the mechanism and functional impact of protein kinase C activation on sst2A receptors. In this report, we examined the involvement of protein kinase C in homologous and heterologous sst2A receptor phosphorylation, identified the regions of the receptor phosphorylated in response to agonist and PMA, and determined the effect of PKC activation on receptor internalization.
**EXPERIMENTAL PROCEDURES**

**Hormones and Supplies—**Cell culture media and G418 were purchased from Life Technologies, Inc. and fetal bovine serum was from JRH Biosciences (Lexiexa, KS). The generation and specificity of the sst2A receptor antisera (R2–88) has been described (18). Leupeptin, pepstatin A, phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, bacitracin, PMA, N-chlorosuccinimide, cytochrome c oxidase from Sigma, N-dodecyl-β-maltoside was purchased from Calbiochem. Pertussis toxin was purchased from List Biological Laboratories, Inc. (Campbell, CA). Okadaic acid and GF109203X-HCl were purchased from LC Laboratories (Woburn, MA). Dowex AG 1-8X anion exchange resin (200–400 mesh, chloride form), Bradford reagent, and reagents for electrophoresis and Western blotting were obtained from Bio-Rad. Phosphate-free DMEM and (P)Hosphohistone were purchased from ICN Biomedicals (Irvine, CA). [3H]Inositol (specific activity, 18.9 Ci/mmol) was from Amersham Pharmacia Biotech. All other reagents were of the best grade available and were purchased from commercial sources.

**Cell Culture—**The clonal GH4-R2.20 cell line, hereafter referred to as GH-R2 cells, was generated by transfecting GH4-C1 pituitary tumor cells with the rat sst2A receptor and was maintained in DMEM/F12 medium containing 10% newborn calf serum as described previously (13). Experimental cultures were used 2–7 days after seeding, with a medium change 18–24 h prior to use. [32P]O-labeling experiments were carried out with cells plated in 100-mm dishes, whereas receptor binding experiments used cells plated in 35-mm wells.

**Measurement of Inositol Phosphates—**GH-R2 cells were seeded at a density of 2 × 10^5/5-mm plate and fed 2 days later with DMEM/F12 containing 10% newborn bovine serum. The cells were then labeled with [3H]inositol-deficient DMEM containing 10% dialyzed fetal bovine serum for 24 h. The cells were washed twice with 1 ml HBSS buffer (118 mM NaCl, 4.6 mM KCl, 0.5 mM CaCl2, 1.0 mM MgCl2, 150 mM NaCl, pH 7.4) containing protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 0.1 mM sodium orthovanadate, and 100 nM okadaic acid). Following centrifugation, the cell pellet was resuspended in 1 ml of lysing buffer (150 mM N H4PO4, 150 mM NaCl, pH 7.4) containing protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 0.1 mM sodium orthovanadate, and 100 nM okadaic acid). Following centrifugation, the cell pellet was resuspended in 1 ml of homogenization buffer (10 mM Tris-HCl, 5 mM EDTA, 3 mM EGTA, pH 7.6) containing phosphatase inhibitors and incubated on ice for 15 min. Following homogenization and a two step centrifugation procedure, membranes were resuspended in cold gly-gly buffer (20 mM glyc Glycine, 1 mM MgCl2, 250 mM sucrose, pH 7.2), snap frozen, and stored at −70°C until assay.

**Purification of the Phosphorylated sst2A Receptor—**Metabolic labeling of cells and subsequent immunoprecipitation of the sst2A receptor was carried out as described previously (13). Briefly, cells were incubated for 3 h in phosphate-free DMEM containing 1 μCi of [32P]orthophosphate and 1% newborn calf serum. Following treatment with various hormones or pharmacological agents, cells were cooled, washed, and resuspended into cold HEPES-buffered saline (150 mM NaCl, 20 mM Heps, pH 7.4) containing protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml sodium pyrophosphate, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, 50 μg/ml bacitracin, 5 mM EDTA, 3 mM EGTA, pH 7.6) containing protease inhibitors and incubated on ice for 15 min. Following homogenization and a two step centrifugation procedure, membranes were resuspended in cold gly-gly buffer (20 mM glyc Glycine, 1 mM MgCl2, 250 mM sucrose, pH 7.2), snap frozen, and stored at −70°C until assay.

**Chemical Cleavage and Peptide Mapping of Phosphorylated sst2A Receptor—**Phosphorylated receptor, immunoprecipitated from [32P]-labeled GH-R2 cells, was located on dried SDS acrylamide gels by autoradiography. The dried gel piece containing the receptor was cut out and rehydrated for 10 min, and the receptor was eluted by incubating the chopped gel in 1 ml of elution buffer (50 mM NH4HCO3 buffer, pH 7.8, 0.1% SDS (w/v), 0.5% 2-mercaptoethanol (v/v) overnight at 30°C. The eluted receptor was then dialyzed against 12% trichloroacetic acid using 20 μg of boiled RNase A as carrier as described previously (20). For cleavage at methionine residues, the precipitated receptor was dissolved in 50 μl of 70% formic acid and incubated with 100 mg/ml cytochrome c (CNBr) for 2 h at room temperature (21). The sample was then frozen on dry ice and lyophilized. For cleavage at aspartic and glutamic acid residues, the sample was incubated with N-chlorosuccinimide (NCS) using a modification of the method described by Lischwe and Ochs (22). The precipitated receptor was dissolved in 20 μl of urea, glacial acetic acid, and water (1:1:1 ml:ml) and denatured by incubating at room temperature for 30 min. Following the addition of 20 μl of 50 μl NCS in urea, glacial acetic acid, and water, the sample was incubated for 30 min at room temperature. Another 20 μl of 50 μl NCS in urea, glacial acetic acid, and water is then added, and the incubation is continued for an additional 30 min. Following the addition of 1 ml of cold elution buffer, peptides were precipitated with trichloroacetic acid as described above.

**Phosphopeptides generated with CNBr or NCS were separated on a discontinuous Tricine-urea SDS-PAGE system described by Schagger and von Jagow (23) using a 16.5% acrylamide, 6% urea resolving gel.** Following electrophoresis, the phosphopeptides are electrophoretically transferred to polyvinylidene difluoride membrane as described previously (13) and analyzed using a PhosphorImager (Molecular Dynamics) (19). In some experiments, the C-terminal receptor peptide was identified by immunoblotting (13) with the R2–88 sst2A receptor antiserum (1:10,000) (18).

**Radioligand Binding and Internalization—**The somatostatin analog Tyr11SRIF (Bachem, Torrance, CA) was radioiodinated using chloramine T and subsequently purified by reverse-phase high performance liquid chromatography. Internalization of the receptor-bound ligand was examined using two experimental approaches differing in the temperature of radioligand binding. In both paradigms, GH-R2 cells were washed with fresh buffer containing 150 mM NaCl, 5 mM Hepes, pH 7.4, and 5 mg/ml lactalbumin hydrolysate) and incubated in the absence or presence of various pharmacological agents for the times indicated. In one paradigm, approximately 150,000 cpm of [32P]-Tyr11SRIF was added either without or with 100 nM unlabeled SRTI, and the incubation was continued at 37°C for various times. Alternatively, following incubation with the pharmacological agents, the cells were washed with cold binding buffer and incubated at 4°C for 2 h in fresh buffer containing [32P]-Tyr11SRIF (150,000 cpm/million) without or with 100 nM unlabeled SRIF, conditions in which equilibrium binding to cell surface receptors is achieved. Following the binding reaction, the cells were washed with cold buffer to remove unbound trace and then incubated for various times at 37°C in the continued absence or presence of pharmacological agents to allow internalization of the receptor-bound ligand. Following internalization at 37°C, cells were rinsed with cold binding buffer and then incubated for 5 min in cold acidic glycine-buffered saline (100 mM glycine, 50 mM NaCl, pH 3.0) to release a surface-bound ligand (13). After collecting the acidic buffer, cells were dissolved in 0.1 N NaOH. The radioactivity in both the glycine buffer (representing total binding) and the cell lysates (representing internalized ligand) was then measured in an Amersham Pharmacia Biotech gamma spectrometer at an efficiency of 75%. Specific binding was calculated as the difference between the amount of radioligand bound in the absence (total binding) and presence of 100 nM SRIF (nonselective binding).

**Other Methods—**Protein A (Sigma) was covalently coupled to CNBr-activated Sepharose B according to the manufacturer’s instructions (Amersham Pharmacia Biotech). Receptor phosphorylation was quantitated using a PhosphorImager (Molecular Dynamics) (19). Unless otherwise indicated results of a representative experiment are shown; all experiments were performed at least two times.

**RESULTS**

**Effect of Protein Kinase C Activation on sst2A Receptor Phosphorylation—**Our previous studies showed that incubation of GH-R2 cells with the protein kinase C activator PMA markedly stimulated sst2A receptor phosphorylation (13). To determine whether this pathway was of physiological significance, we examined the effect of two hormones previously shown to activate phospholipase C in the parental line used to generate...
GH-R2 cells, namely GH4C1 cells. Surprisingly, incubation with 100 nM TRH did not stimulate sst2A receptor phosphorylation in GH-R2 cells (data not shown). However, further investigation showed that TRH did not increase inositol phosphate formation in this cell line (Table I). Because bombesin did induce a modest increase in inositol phosphate accumulation (Table I), we next incubated 32PO4-labeled cultures with this peptide. Following detergent solubilization and partial purification by lectin chromatography, the sst2A receptor was immunoprecipitated with a specific receptor antibody and analyzed by SDS-PAGE, autoradiography, and phosphoimaging. As shown in Fig. 1, bombesin caused a time-dependent increase in sst2A receptor phosphorylation, which reached 1.8 ± 0.1 times the basal level after 5 min. Although this increase in phosphorylation was considerably less than that produced by a 5-min incubation with agonist or PMA (7.3 ± 0.9 and 5.7 ± 0.5 fold, respectively), the observation that bombesin rapidly stimulated sst2A receptor phosphorylation showed that cross-talk occurs between the bombesin and sst2A receptors.

Because bombesin produced a rather modest increase in sst2A receptor phosphorylation, we further characterized the more robust PMA response. To this end, 32PO4-labeled GH-R2 cells were incubated with 200 nM PMA for various periods of time (Fig. 2, left panel) or with different concentrations of PMA for 15 min (Fig. 2, right panel). PMA-stimulated receptor phosphorylation was half-maximal at about 5 min, maximal by 15 min, and maintained through 30 min of incubation (Fig. 2, left panel). Increased receptor phosphorylation was evident upon incubation with 5 nM PMA and was concentration-dependent (EC50 ~ 50 nM) reaching a maximum at 100 nM PMA (Fig. 2, right panel). Thus, sst2A receptor phosphorylation depends on both the concentration and duration of PMA exposure.

The Role of Protein Kinase C in sst2A Receptor Phosphorylation—We used two different approaches to assess the role of PKC in agonist- and PMA-induced sst2A receptor phosphorylation. To determine whether the sst2A receptor was coupled to phospholipase C in GH-R2 cells, we measured the effect of the sst2 receptor selective analog, SMS 201–995 (SMS) on [3H]inositol phosphate accumulation. SMS produced a small, but reproducible increase in IP formation (Table I), suggesting that it could lead to PKC activation in GH-R2 cells.

We next assessed the role of PKC in PMA and agonist-dependent sst2A receptor phosphorylation using the selective PKC inhibitor GF109203X (24). 32PO4-labeled GH-R2 cells were preincubated in the presence or absence of 4 μM GF109203X for 15 min prior to a 5-min incubation with no additions, 100 nM SMS, or 200 nM PMA. The data in Fig. 3 show that GF109203X abolished PMA-induced sst2A receptor phosphorylation but had no effect on SMS stimulation. Bombesin-stimulated receptor phosphorylation was also blocked by GF109203X (data not shown). Thus PMA- and bombesin-stimulated sst2A receptor phosphorylation are mediated by activation of PKC, whereas agonist-stimulated phosphorylation is not.

Despite the differences in mechanism, phosphorylation in response to PMA and SRIF may occur at common sites on the receptor. With the expectation that two agents, which produced receptor phosphorylation at identical sites, should not have an additive effect, we measured the increase in sst2A receptor phosphorylation produced by maximal concentrations of both
immunoprecipitated from cells treated with 100 nM SRIF (gen-
A). CNBr cleavage of the sst2A receptor nine residues (Fig. 5
encompass intracellular regions containing serine and threo-
ne and, to a small extent, on threonine residues (13). However,
phosphorylation but could not distinguish phosphorylation
within the C-terminal and the third intracellular receptor
domains.

To discriminate between the third intracellular loop and the
C-terminal regions of the receptor, we utilized NCS to hydro-
lyze the receptor protein at tryptophan residues (22). Cleavage
of the sst2A receptor with NCS is predicted to generate nine
peptides, five of which represent intracellular regions of the
receptor containing serine and threonine residues (13). However,
to determine the functional consequences of sst2A receptor
phosphorylation, the phosphorylation sites on the receptor
must first be identified. We therefore used peptide mapping to
characterize the intracellular regions of the sst2A receptor that
were phosphorylated.

Chemical cleavage of the receptor at methionine residues
with CNBr is predicted to generate 11 peptides, four of which
encompass intracellular regions containing serine and threo-
ine residues (Fig. 5A). CNBr cleavage of the sst2A receptor
immunoprecipitated from cells treated with 100 nM SRIF gen-
erated a single phosphorylated band between 8 and 9 kDa (Fig.
6, top panel). Based on the predicted molecular masses of the
expected peptide products, this band could contain peptides
from either the third intracellular loop (8924 Da) or the C-
terminal tail of the receptor (8509 Da). We did not detect
phosphopeptides at molecular masses predicted for either the
C-terminal peptide from the first intracellular loop (2197 Da)
or the peptide from the second intracellular loop (2980 Da).
CNBr cleavage of a basally phosphorylated receptor or receptor
phosphorylated in response to treatment with 200 nM PMA also
produced a single phosphorylated band between 8 and 9 kDa
(data not shown).

To directly localize the C-terminal tail receptor peptide, we
electrophoretically transferred the CNBr-generated cleavage
products to polyvinylidene difluoride membrane and then
immunoblotted with the R2–88 receptor antibody, which recog-
nizes a region in the C terminus of the sst2A receptor (18). As
can be seen in Fig. 6 (top panel), a single immunoreactive
peptide was detected at the expected molecular weight. Fur-
ther, the CNBr-generated immunoreactive receptor peptide co-
migrated with the phosphorylated band. Thus, Western blot
analysis of CNBr cleavage products confirmed that the C-
terminal tail of the receptor was a potential site for sst2A receptor
phosphorylation but could not distinguish phosphorylation
within the C-terminal and the third intracellular receptor
domains.

Effect of PKC Activation on sst2A Receptor-Ligand Internal-
ization—To investigate the functional consequences of PKC-

![Figure 3](image3.png)  ![Figure 4](image4.png)

**Fig. 3.** The effect of protein kinase C inhibition on agonist-
and PMA-stimulated receptor phosphorylation. 32PO4-labeled
GH-R2 cells were incubated in the absence or presence of 4 μM
GF109203X for 15 min prior to the addition of 100 nM SMS, 200 nM
PMA, or a no agent. Following an additional 5 min of incubation, the
sst2A receptor was purified and analyzed by SDS-PAGE and phos-
phoimaging. The top panel shows an autoradiogram from a repre-
sentative experiment. The bottom panel shows the amount of receptor phosphorylation measured in two independent experiments by phoshoimaging (mean ±
range).

**Fig. 4.** Additivity of sst2A receptor phosphorylation in re-
response to agonist and PMA. 32PO4-labeled GH-R2 cells were incu-
bated either with no additions, 100 nM SRIF for 15 min, 200 nM PMA for
20 min, or PMA for 5 min followed by both PMA and SRIF for the
subsequent 15 min. After detergent solubilization and purification
by lectin chromatography and immunoprecipitation, proteins were ana-
yzed by SDS-PAGE followed by either autoradiography (top) or phos-
phoimaging (bottom, mean ± range of two independent experiments).
mediated receptor phosphorylation, we assessed the cellular distribution of receptor-bound ligand following pretreatment of GH-R2 cells with PMA (Fig. 7). In one type of experiment (Fig. 7, left panels), GH-R2 cells were preincubated with 200 nM PMA for 15 min at 37 °C, prior to the addition of $[^{125}\text{I}]-\text{Tyr}^{11}\text{SRIF}$. Following continued incubation at 37 °C for the times shown, cells were chilled and then treated with cold acidic glycine-buffered saline to release surface-bound ligand. After collecting the acidic wash, the cells were dissolved in base acidic glycine-buffered saline to release surface-bound ligand. Following detergent solubilization, lectin affinity chromatography, and immunoprecipitation, proteins were subjected to SDS-PAGE. Receptor was localized by autoradiography and then eluted from the gel as described under “Experimental Procedures.” Eluted protein was hydrolyzed with either 100 mg/ml CNBr or 50 mM NCS, and the resulting peptides were resolved by Tricine-SDS-PAGE. Following transfer to a polyvinylidene difluoride membrane, phosphopeptides were detected by phosphoimaging (right). The C-terminal receptor peptide was subsequently identified by immunoblot analysis (left).

In one type of experiment (Fig. 7, left panels), GH-R2 cells were preincubated with 200 nM PMA for 15 min at 37 °C, prior to the addition of $[^{125}\text{I}]-\text{Tyr}^{11}\text{SRIF}$. Following continued incubation at 37 °C for the times shown, cells were chilled and then treated with cold acidic glycine-buffered saline to release surface-bound ligand. After collecting the acidic wash, the cells were dissolved in base acidic glycine-buffered saline to release surface-bound ligand. Following detergent solubilization, lectin affinity chromatography, and immunoprecipitation, proteins were subjected to SDS-PAGE. Receptor was localized by autoradiography and then eluted from the gel as described under “Experimental Procedures.” Eluted protein was hydrolyzed with either 100 mg/ml CNBr or 50 mM NCS, and the resulting peptides were resolved by Tricine-SDS-PAGE. Following transfer to a polyvinylidene difluoride membrane, phosphopeptides were detected by phosphoimaging (right). The C-terminal receptor peptide was subsequently identified by immunoblot analysis (left).

Peptide fragments expected from cleavage of the sst2A receptor by CNBr and NCS. The structure of the rat sst2A receptor is shown schematically with serine and threonine residues in the intracellular regions designated by filled circles. Cleavage with CNBr or NCS results in hydrolysis of proteins at methionine or tryptophan residues, respectively. Cleavage of the rat sst2A receptor with CNBr (top) is predicted to generate a terminal methionine and eleven peptides, four of which contain potential intracellular phosphate acceptor sites. Cleavage of the receptor with NCS (bottom) is predicted to generate nine peptides, five of which contain intracellular serines or threonines. Tables show the predicted molecular masses of potential phosphopeptides for each cleavage method.

| Peptide | Predicted M.Wt (Da) |
|---------|---------------------|
| 1st loop-C | 2197 |
| 2nd loop | 2980 |
| 3rd loop | 8924 |
| C-tail | 8509 |

| Peptide | Predicted M.Wt (Da) |
|---------|---------------------|
| 1st loop | 10423 |
| 2nd loop-N | 5057 |
| 2nd loop-C | 1698 |
| 3rd loop | 7409 |
| C-tail | 11030 |

was more than half-maximal. In two experiments there was 5.9 ± 0.25-fold more radioligand inside PMA-treated cells at 2 min than in untreated cells. Overall, PMA dramatically increased both the rate and extent of $[^{125}\text{I}]-\text{Tyr}^{11}\text{SRIF}$ accumulation in cells and reduced the lag between radioligand binding at the cell surface and internalization (Fig. 7, left panels).

In an alternate approach, cells were pretreated with or without 200 nM PMA as described above but the subsequent binding of $[^{125}\text{I}]-\text{Tyr}^{11}\text{SRIF}$ was carried out at 4 °C so that the receptor-ligand complex remained at the cell surface during the binding incubation (Fig. 7, right panels). Following removal of the bound ligand in the medium, cells were incubated at 37 °C in the continued absence or presence of PMA to allow redistribution of the receptor-ligand complex. At the times shown, the surface-bound and internalized radioligand were measured as described above. In this experimental paradigm, the rate of ligand binding is separated from the measurement of internalization rates, because the amount of radioligand prebound to the receptor is unaffected by the PMA pretreatment. Again, there was a time-dependent accumulation of the sst2A receptor-ligand complex in the intracellular compartment (Fig. 7A, right top panel), and this accumulation was paralleled by a decrease in surface binding (Fig. 7A, right bottom panel). PMA dramatically stimulated the internalization of the receptor-ligand complex (Fig. 7, right panels). The effect was greatest at early time points; at 2 min there was 6.9 ± 1.1-fold ($n = 3$) more internalized radioligand in PMA-treated than in untreated cells. Together, these data demonstrate that incubation of GH-R2 cells with PMA markedly stimulates both the initial rate and extent of sst2A receptor-mediated internalization.

To determine whether the effect of PMA on internalization occurred through activation of PKC, we preincubated cells in the presence or absence of the selective PKC inhibitor, GF 109203X for 15 min prior to a 5-min incubation with no additions or 200 nM PMA (Fig. 8). Under these conditions, GF 109203X completely inhibits PMA-stimulated sst2A receptor phosphorylation with no effect on phosphorylation of the receptor in response to agonist (Fig. 3). Cells were then chilled and incubated at 4 °C for 2 h with $[^{125}\text{I}]-\text{Tyr}^{11}\text{SRIF}$ and then warmed and incubated at 37 °C in the continued absence or presence of 4 μM GF 109203X and PMA to allow internalization to occur. PMA exposure again stimulated the intracellular ac-
cumulation of the receptor-ligand complex. Although GF109203X did not significantly affect $^{[125I]}$Tyr11-SRIF internalization in control cells, it abolished the increase in sst2A receptor internalization in response to the phorbol ester (Fig. 8).

Mechanisms of sst2A Receptor Internalization—Pertussis toxin pretreatment prevents sst2A-mediated inhibition of adenylyl cyclase but does not affect sst2A receptor phosphorylation (13). To assess the requirement for sst2A receptor Gi/o coupling for receptor internalization, we pretreated GH-R2 cells in the absence or presence of 100 ng/ml pertussis toxin (PTX) for 18–24 h and then measured the internalization of prebound $^{[125I]}$Tyr11-SRIF. This PTX treatment prevented SMS inhibition of vasoactive intestinal peptide-stimulated adenylyl cyclase activity (data not shown). Control and PTX-treated cells were incubated at 37 °C for 15 min in the absence or presence of 200 nM PMA and then at 4 °C for 2 h with $^{[125I]}$Tyr11-SRIF. The amount of internalized ligand was determined following a 5-min incubation at 37 °C in the continued absence or presence of PMA. PTX treatment had no effect on the internalization of the receptor-bound ligand in either the absence or in the presence of PMA (Fig. 9, top panel). We therefore conclude that coupling of the sst2A receptor to PTX-sensitive G proteins is not required for receptor internalization nor does receptor uncoupling alter PMA stimulation of internalization.

To examine the role of clathrin-coated pits in $^{[125I]}$Tyr11-SRIF internalization, cells were preincubated with or without PMA as described above, chilled, and incubated with $^{[125I]}$Tyr11-SRIF 4 °C for 2 h in the presence or absence of 0.45 M sucrose, which disrupts endocytosis via clathrin-coated pits (25). The cells were then washed, warmed to 37 °C, and incubated in the continued absence or presence of PMA and sucrose. The surface-bound and internalized ligand was measured after a 5-min incubation. Exposure to hypertonic sucrose markedly inhibited $^{[125I]}$Tyr11-SRIF internalization in both untreated cells and PMA-stimulated cells (Fig. 9, bottom panel), indicating that both basal- and PMA-stimulated internalization of the sst2A receptor occurs through clathrin-coated pits.

Taken together, these data show that sst2A receptor internalization in response to agonist, either alone or in the presence of PMA and sucrose. The surface-bound and internalized ligand was measured after a 5-min incubation. Exposure to hypertonic sucrose markedly inhibited $^{[125I]}$Tyr11-SRIF internalization in both untreated cells and PMA-stimulated cells (Fig. 9, bottom panel), indicating that both basal- and PMA-stimulated internalization of the sst2A receptor occurs through clathrin-coated pits.

**DISCUSSION**

A number of early studies reported modulatory effects of protein kinase C on SRIF receptor signaling and binding. Acute exposure to phorbol 12-myristate 13-acetate was shown to attenuate SRIF inhibition of adenylyl cyclase in both S49 lymphoma cells (26) and GH4C1 pituitary tumor cells (27). Protein kinase C activation also blocked SRIF-inhibition of Ca$^{2+}$ currents in chick and rat sympathetic neurons (28, 29). Treatment with phorbol esters for several hours decreased SRIF binding in GH4C1 pituitary cells (30), pancreatic acinar cells (31, 32), and gastric chief cells (33). In GH4C1 cells, TRH, which increases diacylglycerol formation and PKC activity, led to a similar down-regulation of SRIF receptors as did phorbol esters (34). In chief cells protein kinase C activation with cholecystokinin also decreased SRIF receptor binding (33). However, such heterologous regulation of SRIF receptors was not observed in

**FIG. 7.** The effect of PMA on the internalization of the sst2A receptor-ligand complex. Left, following a 15-min incubation at 37 °C in the presence (○) or absence (○) of 200 nM PMA, GH-R2 cells were further incubated with $^{[125I]}$Tyr11-SRIF (150,000 cpm/ml) at 37 °C for the times indicated. Right, after a 15-min treatment at 37 °C with (○) or without (○) 200 nM PMA, GH-R2 cells were incubated with $^{[125I]}$Tyr11-SRIF (150,000 cpm/ml) at 4 °C for 2 h. Cells were then washed to remove unbound $^{[125I]}$Tyr11-SRIF, warmed, and further incubated at 37 °C in the continued presence or absence of PMA. In both panels, cells were chilled at the times shown, washed to remove unbound peptide, and incubated for 5 min at 4 °C with acidic glycine-buffered saline to release surface-bound ligand. Following removal of the glycine buffer, the cells were dissolved in 0.1 N NaOH. Radioactivity was measured in both the cell lysates, representing internalized ligand (top) and in the acid wash representing surface-bound ligand (bottom). Data represent the specific binding (mean ± S.E.) in triplicate samples in a representative of three independent experiments.
the addition of 200 nM PMA to some of the dishes (○, ■), cells were incubated for an additional 5 min at 37 °C. Cells were then incubated at 4 °C for 2 h with [125I-Tyr11]SRIF (150,000 cpm/ml) in the absence and presence of 100 nM SRIF, washed, and then incubated at 37 °C in the continued presence or absence of GF109203X and PMA to allow receptor internalization to occur. At the times shown, cells were chilled and washed with acidic glycinum-buffered saline to remove surface-bound ligand and then dissolved in 0.1 N NaOH. The graph shows the amount of specifically bound radioligand in the internalized compartment (mean ± S.E. of triplicate samples in one of two independent experiments).

FIG. 8. The effect of protein kinase C inhibition on PMA-stimulated internalization. GH-R2 cells were incubated in the presence (■, ■) or absence (○, ○) of 4 μM GF109203X at 37 °C for 15 min. After the addition of 200 nM PMA to some of the dishes (▲, ▲), cells were incubated for an additional 5 min at 37 °C. Cells were then incubated at 4 °C for 2 h with [125I-Tyr11]SRIF (150,000 cpm/ml) in the absence and presence of 100 nM SRIF, washed, and then incubated at 37 °C in the continued presence or absence of GF109203X and PMA to allow receptor internalization to occur. At the times shown, cells were chilled and washed with acidic glycinum-buffered saline to remove surface-bound ligand and then dissolved in 0.1 N NaOH. The graph shows the amount of specifically bound radioligand in the internalized compartment (mean ± S.E. of triplicate samples in one of two independent experiments).

PKC-stimulated sst2A Receptor Phosphorylation and Internalization

We had previously found that incubation of GH-R2 cells with PMA for 15 min causes a 30-fold increase in sst2A receptor phosphorylation, an effect similar in magnitude to that produced by agonist (13). The studies reported here demonstrate for the first time that PMA increases the internalization of the sst2A receptor-hormone complex concomitantly with receptor phosphorylation. Stimulation of both receptor phosphorylation and endocytosis occurs within minutes of PMA treatment and these PMA effects are both blocked by the protein kinase C inhibitor GF109203X. Further, 32P3O4 incorporation into the C-terminal tail and the third intracellular loop of the sst2A receptor occurs with PMA as well as in receptor treatment of GH-R2 cells. Hence, SRIF and PMA both lead to receptor phosphorylation at multiple sites.

Our conclusion that agonist binding leads to phosphorylation of the sst2A receptor within both the C-terminal tail and the third intracellular loop differs from that of Schwartkopf et al. (37) who deduced that agonist-dependent phosphorylation of the sst2A receptor is restricted to the C terminus. Their conclusion was based on the observation that truncation of a T7-tagged sst2A receptor at residue 325, which removes the last 44 amino acids from the C terminus, prevents agonist-induced receptor phosphorylation (37). However, when considered in light of our biochemical data showing that the wild-type receptor is phosphorylated within the third intracellular loop as well as in the C-terminal region, two alternate explanations seem more likely. Receptor phosphorylation may occur in sequential steps such that phosphorylation of residues in the C-terminal tail of the sst2A receptor is required for subsequent phosphorylation of residues in the third intracellular loop. Such a hierarchical phosphorylation scheme has been proposed for the phosphorylation of the N-formylpeptide receptor by GRK2 (38). Alternatively, it is possible that the sst2A receptor kinase interacts most avidly with a receptor domain that is different from the domain phosphorylated, as has been shown for rhodopsin kinase (39). Thus, the Δ325 truncation of the sst2A receptor, rather than removing all phosphorylation sites, may produce conformational changes that indirectly decrease the efficiency of receptor phosphorylation.

The similarity between the SRIF- and PMA-induced phosphorylation of the sst2A receptor led us to investigate whether the two effects were catalyzed by the same enzymes(s). SRIF stimulation of GH-R2 cells led to a modest 60% increase in IP formation (Table I) indicating that SRIF could activate protein kinase C in this cell line. The observed increase in IP formation in GH-R2 cells is consistent with previous reports that sst2A is linked to phosphoinositide hydrolysis when overexpressed in COS-7 (14) and F4 pituitary cells (15). However, the protein kinase C inhibitor GF109203X did not affect SRIF stimulation of sst2A receptor phosphorylation, whereas the PMA stimulation was blocked. Receptor phosphorylation in response to
PKC-stimulated sst2A Receptor Phosphorylation and Internalization

bomabesin, which stimulated IP formation somewhat more than SRIF, was also blocked by GF10920X. Hence, sst2A receptor phosphorylation can occur by different biochemical pathways. Whereas protein kinase C activity is essential for the action of PMA and bombesin, it is not involved in agonist regulation. Why GF10920X does not at least partially inhibit SRIF-stimulated sst2A phosphorylation is unclear. Perhaps the DAG formed upon SRIF stimulation is not sufficient to activate PKC. This possibility is supported by the observation that bombesin, which induced a modest increase in IP formation, elicited only a 2-fold increase in sst2A receptor phosphorylation. Thus the contribution of PKC to agonist-stimulated receptor phosphorylation may be sufficiently small in GH-R2 cells as to be indiscernable. Overall, our studies clearly demonstrate that in the case of sst2A receptor phosphorylation homologous and heterologous regulation occur by different mechanisms in GH-R2 cells. Whether this conclusion can be extended to other cell types remains to be determined.

The enzymatic pathway involved in PMA- and bombesin-stimulated receptor phosphorylation is not known but could involve either direct phosphorylation of the receptor by PKC or PKC activation of a different kinase. Direct sst2A phosphorylation by PKC is possible because PKC consensus sites are present in both the third intracellular loop (KYKSSGIR and RKKEEQK) and the C-terminal tail (RSDSKQDK and RLNETQQR) of the receptor (40). Although PKC catalyzed phosphorylation has been shown to regulate the activity of some GRKs (1, 3), PMA-stimulated sst2A phosphorylation is unlikely to result from GRK activation because PMA increases sst2A receptor phosphorylation in the absence of SRIF, whereas GRKs are thought to phosphorylate only agonist-occupied receptors (1–3). Consistent with GRK phosphorylation of sst2A being dependent on agonist binding, GRK2 translocates to the plasma membrane upon SRIF treatment of S49 lymphoma cells (41), which express the sst2A receptor (42). Further, the observation that SRIF and PMA increase sst2A phosphorylation in an additive manner suggests that different residues are phosphorylated under the two conditions and provides additional support for the conclusion that GRKs do not catalyze both SRIF- and PMA-stimulated sst2A receptor phosphorylation. Several other G protein-coupled receptors, including rhodopsin, are similarly phosphorylated by PKC and GRK at different residues (19, 43). Thus, based on available data, the simplest hypothesis is that PKC directly phosphorylates the sst2A receptor at sites other than those targeted by GRKs.

A number of investigators have shown that SRIF binding leads to the internalization of the hormone-sst2A receptor complex via a clathrin-mediated pathway (5). We show here that PMA dramatically increases this rate of internalization and that the PMA-stimulated endocytosis is also blocked by hyper tonic sucrose, an inhibitor of receptor internalization via clathrin-coated vesicles (25). Several observations indicate that the PMA effects on sst2A receptor phosphorylation and increased receptor internalization are linked. They both occur within minutes of PMA treatment and are both blocked by protein kinase C inhibition. In contrast, endocytosis of the SRIF-receptor complex in the absence of PMA is unaffected by GF10920X. Further, the effect of PMA is specific to sst2A internalization; we did not observe significant stimulation of sst1 receptor endocytosis in transfected GH pituitary cells.2 Similarly, sst3 internalization was not affected by phorbol ester treatment in transfected RIN1046–38 cells (44). Thus, PMA is unlikely to increase sst2A internalization by altering the function of components of the cellular endocytic machinery. How ever, further experiments will be required to establish a causal relationship between PKC-catalyzed sst2A receptor phosphorylation and increased receptor internalization.

Although hormone binding was found to induce sst2A receptor endocytosis in all studies to date, substantial quantitative differences were observed in the extent of internalization at steady state. The fraction of receptor-bound hormone, which was resistant to an acid wash after a 60-min incubation at 37 °C, was about 20% in CHO-K1 cells (45), 50–75% in COS-7 cells (46), and over 95% in HEK cells (37). In GH-R2 cells we have observed anywhere from 20 to 50% internalization at steady state in different experiments (Ref. 13 and this report). Although sst2A receptor internalization will undoubtedly be influenced by the cellular complement of GRKs and arrestins present in each cell line, the PKC stimulation of endocytosis described in this report suggests that variable activation of PKC by either serum factors or by SRIF itself may also affect the rate and extent of sst2A receptor internalization.

The regulation of sst2A receptor function in the pituitary via heterologous activation of PKC is likely to be of substantial physiological importance. The sst2A receptor isotype mediates the effect of SRIF on the secretion of several pituitary hormones, including GH (7, 47), and overall hormone secretion by the pituitary depends on the interactions of multiple hypothalamic and paracrine factors many of which activate protein kinase C. Our data suggest that, in addition to their direct stimulatory effects on pituitary hormone synthesis and secretion, these factors may also blunt the inhibitory effect of SRIF by regulating the cell surface expression of sst2A. In fact, regulation of sst2A receptor trafficking by PKC activation may have physiological ramifications in many other tissues, including the brain, the endocrine and exocrine pancreas, the immune system, and the GI tract. Furthermore, because the diagnostic and therapeutic use of radiolabeled SRIF analogs depends to a large extent on their internalization by sst2A receptors expressed on tumors (48), understanding the mechanisms by which PKC activation regulates sst2A internalization in various cancers and the use of agents which act via PKC to stimulate receptor-mediated endocytosis of radiolabeled SRIF analogs may have important clinical applicability.

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