Agonist-dependent Dissociation of Human Somatostatin Receptor 2 Dimers

A ROLE IN RECEPTOR TRAFFICKING*

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G-protein-coupled receptors (GPCRs) represent the largest and most diverse family of cell surface receptors. Several GPCRs have been documented to dimerize with resulting changes in pharmacology and signaling. We have previously reported, by means of photobleaching fluorescence resonance energy transfer (pbFRET) microscopy and fluorescence correlation spectroscopic analysis in live cells, that human somatostatin receptor (hSSTR) 5 could both homodimerize and heterodimerize with hSSTR1 in the presence of the agonist SST-14. By contrast, hSSTR1 remained monomeric when expressed alone regardless of agonist exposure in live cells. However, the effect of the agonist on other hSSTR members remains unknown. Using pbFRET microscopy and Western blot, we provide evidence for agonist-dependent dissociation of self-associated hSSTR2 stably expressed in CHO-K1 and HEK-293 cells. Furthermore, the dissociation of the hSSTR2 dimer occurred in a concentration-dependent manner. Moreover, blocking receptor dissociation using a cross-linker agent perturbed receptor trafficking. Taking these data together, we suggest that the process of GPCR dimerization may operate differently, even among members of the same family, and that receptor dissociation as well as dimerization may be important steps for receptor dynamics.

G-protein-coupled receptors (GPCRs) represent ~1% of the human genome, an estimate exceeding 800 genes (1). The initial notion that GPCRs are present on the membrane as monomeric entities in a 1:1 stoichiometric ratio with their G-protein has since been reinterpreted. Several studies have shown by using a combination of techniques such as co-immunoprecipitation, bioluminescence resonance energy transfer, and fluorescence resonance energy transfer (FRET) that at least some GPCRs assemble on the membrane as functional homo- and heterodimers (2–4). Dimerization of GPCRs has been shown to affect a multitude of receptor functions, including ligand binding, signaling, receptor desensitization, and receptor trafficking (2–5).

The mechanism by which GPCR dimerization occurs remains obscure and controversial. One model suggests that ligand binding induces a conformational change in the receptor that favors dimer formation. In contrast to this model, the presence of GPCRs, which may be assembled as preformed dimers, has been shown for members of the class C subfamily, which includes GABAergic receptors (6–8), calcium-sensing receptor (9, 10), the metabotropic glutamate receptor (11), and the sweet taste receptors (12–14). This paradigm of GPCR assembly, however, is not consistent among the other families of GPCRs (2). Several reports have shown that agonist plays an active role in GPCR dimerization at the plasma membrane, suggesting an equilibrium between GPCR dimers or monomers that can be regulated by ligand occupancy. These receptors include the human somatostatin receptors (hSSTRs) (15, 16), the dopamine D2 receptor (17), the gonadotropin-releasing hormone receptor (18, 19), the luteinizing hormone/chorionic gonadotropin hormone receptor (20), the bradykinin B2 receptor (21), the thyrotropin-releasing hormone receptor (22), the human cholecystokinin receptor (23), the human thryrotropin receptor (24), the chemokine receptors (25–28), the mouse δ-opioid receptor (δ-OR) (29), and the rhesus neuropeptidase Y4 receptor (30).

We have previously reported that hSSTRs are capable of undergoing both homo- and heterodimerization at the cell membrane (15, 31). Recently, we have demonstrated agonist-dependent homo- and heterodimers on the plasma membrane in live cells using fluorescence correlation spectroscopy (16). One of the receptor subtypes, hSSTR1, did not form homodimers in either the absence or presence of agonist, in contrast to hSSTR5, which showed robust dimerization on agonist exposure. When both receptors were co-expressed in the same cell, two populations of dimers were observed, hSSTR5 homodimers and hSSTR1/hSSTR5 heterodimers (16). The effect of agonist on other members of the hSSTR family is currently unknown.

In the present study using both Western blot and pbFRET analysis, we determined the effect of agonist on hSSTR2 dimerization. We show that agonist-induced a dissociation of preassembled hSSTR2 dimers in a concentration-dependent manner. This effect was inhibited when cell membranes were pretreated with a cross-linking agent. hSSTR2 undergoes internalization on exposure to agonist, however, inhibition of dimer dissociation resulted in impaired receptor internalization. Finally, our data provide evidence suggesting that agonist-

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§ The abbreviations used are: GPCR, G-protein-coupled receptor; pbFRET, photobleaching fluorescence resonance energy transfer; GABA, γ-aminobutyric acid; hSSTR, human somatostatin receptor; δ-OR, mouse δ-opioid receptor; HA, hemagglutinin; FITC, fluorescein isothiocyanate; BS3, bis(sulfosuccinimidyl) suberate; HA-hSSTR, HA-tagged hSSTR; D-PBS, Dulbecco’s phosphate-buffered saline; LTT-SST-28, [Leu²ⁿ-Trp²²,Tyr²⁵] SST-28.
dependent dissociation of self-associated dimers may be a requirement for proper receptor trafficking.

EXPERIMENTAL PROCEDURES

Materials—The peptides SST-14 and [Leu⁴]-p-Tyr³-Tyr²]SST-28 (LTT-SST-28) were purchased from Bachem, Torrance, CA. Fluorescein- and rhodamine-conjugated and non-conjugated mouse monoclonal antibodies against hemagglutinin (HA) (12CA5) were purchased from Roche Applied Science. FITC-conjugated goat anti-mouse affinity-puriﬁed secondary antibody was purchased from Jackson ImmunoResearch Laboratories, West Grove, PA. The cross-linking agent biotin-sulfosuccinimidyl suberate (BS³) was purchased from Sigma.

SSTR Constructs and Expressing Cell Lines—Stable CHO-K1 and HEK-293 cells expressing HA-tagged hSSTR2 were prepared by LipofectAMINE transfection reagent as described previously (15). Stable transfections were made using the vectors pCDNA3.1/Neo (neomycin resistance) from Invitrogen. Clones from CHO-K1 cells were selected and maintained in medium containing ham’s F-12 with 10% fetal bovine serum and 700 μg/ml neomycin. Clones from HEK-293 cells were selected in Dulbecco’s modiﬁed Eagle’s medium containing 10% fetal bovine serum and 700 μg/ml neomycin.

pbFRET Microscopy—pbFRET experiments were performed at the plasma membrane of CHO-K1 cells as described previously (15, 16, 31). In CHO-K1 cells expressing HA-hSSTR2 were treated with different concentrations (0, 10⁻¹⁰, 5 x 10⁻¹⁰, 10⁻⁹ mol/L) of agonist for 10 min at 37°C, fixed with 4% paraformaldehyde in PBS for 20 min on ice, and processed for immunocytochemistry. Immunocytochemistry was performed using monoclonal anti-HA antibodies conjugated to FITC and rhodamine for the donor and acceptor, respectively, before being subjected to pbFRET analysis. The effective FRET efficiency (E) was calculated from the average photobleaching time constant of the donor obtained in the absence (τобр D, A) and presence (τобр D, A) of the acceptor, according to the equation $E = 1 - \left(\frac{\tau_{обр D, A}}{\tau_{обр D, A}}\right) \times 100$.

Western Blot Analysis—Membranes from HA-hSSTR2 stably transfected in HEK-293 cells were prepared using a glass homogenizer in 20 mM Tris-HCl, pH 7.5, as described previously (15). The membrane pellet was washed and resuspended in 20 mM Tris-HCl, pH 7.5, in the absence of dithiothreitol. Membrane protein (50 μg) was treated with SST-14 (0, 10⁻¹⁰, 10⁻⁹, and 10⁻⁸ mol/L) in binding buffer (50 mM Hepes, 2 mM CaCl₂, and 5 mM MgCl₂, pH 7.5) for 30 min at 37°C. When the cross-linking agent BS³ was used, membranes were pretreated with 5 mM BS³ for 1 h at 4°C, and this reaction was terminated by the addition of 50 mM Tris-HCl, pH 7.5, for 15 min at 4°C. After treatment with SST-14, membrane protein was solubilized in Laemmli sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 0.01% bronopol blue, and 710 mM 2-mercaptoethanol (Bio-Rad). The sample was heated at 85°C for various times (0, 15, 30, 60 min) to initiate changes in dimer to monomer formation, we performed Western blot in combination with pbFRET analysis. Western blot was used to confirm whether ligand-activated hSSTR2 is associated with changes in dimer to monomer formation, we performed Western blot.

Membranes from HEK-293 cells stably expressing HA-hSSTR2 (Bmax = 2608 ± 206 fmol/mg of protein, KD = 0.4 ± 0.1 nM) were treated with various concentrations of SST-14 before being processed for Western blot. In the absence of agonist, hSSTR2 was present in both dimer (120 kDa) and monomeric (60 kDa) forms (Fig. 1B). Treatment of hSSTR2 membrane with SST-14 induced a concentration-dependent decrease in dimer formation (Fig. 1B). Increasing concentrations of SST-14 gradually decreased the ratio of hSSTR2 dimers to monomers (Fig. 1C).

RESULTS

Ligand-dependent Dissociation of hSSTR2 Homodimers by pbFRET and Western Blot—CHO-K1 cells were stably expressed with an N-terminal HA-tagged hSSTR2 (Bmax = 435 ± 33 fmol/mg of protein, KD = 0.3 ± 0.1 nM). Cells were treated with different concentrations of SST-14 and processed for hSSTR2 localization performed using pbFRET microscopy. pbFRET microscopy was determined by the photobleaching decay ofonor on the cell surface by using monoclonal anti-HA antibodies conjugated with either fluorescein (donor) or rhodamine (acceptor). As shown in Fig. 1A, in the basal state, the cell surface presented with a high relative FRET efficiency, suggesting the presence of homodimers, compared with SST-14-treated cells. Treatment with SST-14 induced a concentration-dependent decrease in FRET efficiency from a maximum of 11.7 ± 1.1% under basal conditions to 2.1 ± 0.9% with 1 μM SST-14 (Table I and Fig. 1A). Our data indicate that unlike hSSTR1 and hSSTR5, hSSTR2 is self-associated and dissociates on agonist activation. Because FRET is sensitive to distance, changes in FRET efficiency can be perceived as either dissociation of dimers or changes in receptor conformation. To confirm whether ligand-activated hSSTR2 is associated with changes in dimer to monomer formation, we performed Western blot.

Membranes from HEK-293 cells stably expressing HA-hSSTR2 (Bmax = 2608 ± 206 fmol/mg of protein, KD = 0.4 ± 0.1 nM) were chosen for their higher expression levels, were treated with various concentrations of SST-14 before being processed for Western blot. In the absence of agonist, hSSTR2 was present in both dimer (120 kDa) and monomeric (60 kDa) forms (Fig. 1B). Treatment of hSSTR2 membrane with SST-14 induced a concentration-dependent decrease in dimer formation (Fig. 1B). Increasing concentrations of SST-14 gradually decreased the ratio of hSSTR2 dimers to monomers (Fig. 1C). Dissociation of the dimeric complex could be prevented when membranes were pretreated with the cross-linking agent BS³ (Fig. 1D). Western blot in combination with pbFRET analysis supports the notion that hSSTR2 is a self-associated dimer under basal conditions, but on ligand activation, it preferentially dissociates into monomers. It is unclear where there was an appreciable amount of monomeric receptor found by Western blot. One reason could be the result of a solubilization artifact, because receptor dimers can be affected by the concentration of detergent used. Another reason could be the higher expression levels achieved in HEK-293 cells compared with those in CHO-K1 cells used to perform Western blot. Perhaps the monomeric receptors represent an immature intracellular pool that is only functional when the receptors are in dimeric form and are present at the cell surface. With the exception of the...
Because BS3 is a hydrophilic lipid impermeable cross-linking agent and was shown to inhibit agonist-induced hSSTR2 dissociation (Fig. 1D), only dimeric receptors at the cell surface would be affected on treatment. Under basal conditions, hSSTR2 was mainly localized at the cell surface with little or no cytoplasmic localization of receptor in permeabilized cells (Fig. 2A, left panels). Treatment with SST-14 (1 μM) resulted in the internalization of hSSTR2 with decreases in cell surface expression (Fig. 2A, middle panels). Internalization of receptor was appreciably inhibited when cells were treated with the cross-linker prior to the exposure of SST-14 (Fig. 2A, right panels). Thus, we demonstrate that internalization of hSSTR2 can be inhibited when receptor dissociation is blocked. To determine whether receptor internalization was either completely blocked or delayed, we measured internalization kinetics.

Cells expressing hSSTR2 were treated with or without 1 mM cross-linker BS3 to determine the effect of inhibiting receptor dissociation on the kinetics of internalization. We used the radioligand 125I-LIT-SST-28, an agonist to hSSTR2, to measure internalization kinetics. Receptor binding was not inhibited on treatment with BS3. A maximum internalization was achieved at ~30 min, when measured up to 1 h. This internalization rate was significantly altered when cells were pretreated with BS3, consistent with the immunocytochemistry results that inhibition of dimer dissociation impairs receptor trafficking (Fig. 2B).

**DISCUSSION**

The mechanisms underlying dimerization have become paramount in the elucidation of GPCR function. There are two general models that have been proposed for the dimerization of GPCRs that have resulted in contentious debate (for reviews see Refs. 2–4). One model states that GPCRs are preassembled dimeric complexes occurring early following their synthesis in the endoplasmic reticulum, without any ensuing effect on ligand treatment in altering the dimeric state of the receptor. An example that has been well documented in this regard is the GABAB receptor, a relative of the class C subfamily of GPCRs.

**Experimental Procedures.**

**Materials and Methods.**

CHO-K1 cells expressing HA-hSSTR2 were treated with the indicated concentrations of SST-14 and subjected to pbFRET analysis as described under “Materials and Methods.” D – A and D + A, correspond to donor in the absence and presence of acceptor, respectively; τavg, mean of n photobleaching time constants; n, number of cells analyzed; E, average effective FRET efficiency. Means ± S.E. represent three independent experiments.

| SST-14 concentration | τavg  | n    | E    |
|----------------------|-------|------|------|
| Control              | D – A | 13.6 ± 0.3 | 48 | 11.7 ± 1.1 |
| 10^{-10}             | D + A | 15.4 ± 0.3 | 48 | 5.7 ± 1.3  |
| 5 × 10^{-9}          | D – A | 13.2 ± 0.3 | 48 | 2.9 ± 1.2  |
| 10^{-7}              | D + A | 14.0 ± 0.4 | 48 | 2.1 ± 0.9  |
| 10^{-6}              | D – A | 14.1 ± 0.3 | 48 | 1.4 ± 1.1  |
| 10^{-5}              | D + A | 14.3 ± 0.3 | 48 | 1.4 ± 1.1  |
| 10^{-4}              | D – A | 14.2 ± 0.4 | 48 | 2.1 ± 0.9  |
| 10^{-3}              | D + A | 14.5 ± 0.2 | 48 | 2.1 ± 0.9  |

δ-OR (29), little has been shown to support the functional significance for the dissociation of GPCRs. To determine whether dissociation of the dimeric complex is associated with changes in receptor properties, we proceeded to investigate receptor internalization.

**Internalization of hSSTR2**—CHO-K1 cells stably expressing HA-hSSTR2 were grown on coverslips and treated with 1 μM SST-14 for 30 min with or without prior treatment of BS3. Because BS3 is a hydrophilic lipid impermeable cross-linking agent and was shown to inhibit agonist-induced hSSTR2 dissociation (Fig. 1D), only dimeric receptors at the cell surface would be affected on treatment. Under basal conditions, hSSTR2 was mainly localized at the cell surface with little or no cytoplasmic localization of receptor in permeabilized cells (Fig. 2A, left panels). Treatment with SST-14 (1 μM) resulted in the internalization of hSSTR2 with decreases in cell surface expression (Fig. 2A, middle panels). Internalization of receptor was appreciably inhibited when cells were treated with the cross-linker prior to the exposure of SST-14 (Fig. 2A, right panels). Thus, we demonstrate that internalization of hSSTR2 can be inhibited when receptor dissociation is blocked. To determine whether receptor internalization was either completely blocked or delayed, we measured internalization kinetics.

**FIG. 1.** Agonist-dependent dissociation of the hSSTR2 dimer. CHO-K1 cells stably expressing HA epitope-tagged hSSTR2 were subjected to pbFRET analysis using anti-HA monoclonal antibodies conjugated with either FITC (donor) or rhodamine (acceptor) as described under “Experimental Procedures.” Control represents the FRET efficiency of cells that have not been treated with SST-14. Cells were treated with SST-14 for 10 min before being processed for pbFRET microscopy. Means ± S.E. represent three independent experiments, each taken from ~40–50 cells. B, HEK-293 cells stably expressing HA epitope-tagged hSSTR2 were treated with the indicated concentrations of SST-14 for 30 min before being processed and separated on a SDS-7.5% polyacrylamide gel. The represented blot is taken from three independent runs. C, immunoblots from B were quantified for changes in the dimeric:monomeric ratio after SST-14 treatment by densitometry using FluorChem software. D, immunoblot from membranes of HEK-293 cells stably expressing HA-hSSTR2 incubated with 5 mM BS3 prior to treatment with or without 1 μM SST-14. Membranes were separated on a SDS-7% polyacrylamide gel. The immunoblot is a representation of three independent runs.
in stably transfected cells, or the species of receptor used is uncertain. In a recent report, transiently transfected cells resulted in the formation of mostly dimeric immature intracellular human lutropin receptors that exhibited no effect on ligand treatment (34). When stably transfected, however, mature cell surface monomeric receptors were mainly present and displayed ligand-induced increases in dimer formation. Therefore, it was suggested that these promiscuous interactions were occurring at the level of the endoplasmic reticulum in transiently transfected cells because of the accumulation of immature receptors (34).

We have previously reported that hSSTR2 can both homodimerize with hSSTR1 and dopamine 2 receptor in a ligand-dependent manner in stably transfected cells (15, 16, 31), but the functional significance of these observations remains unclear. Dimerization of the β2-adrenergic receptor was suggested to be a requirement for receptor activation (35). Furthermore, using a coumerycin-gyrase B-induced dimerization system on the platelet-activating factor receptor, coumerycin-induced dimerization was sufficient to cause the desensitization and internalization of platelet-activating factor receptor in an agonist-independent fashion (36). Taken together this would suggest that dimerization plays a key role in receptor activation and internalization, although this has not been exemplified for all GPCRs.

In the present study, hSSTR2 was found to be a self-associated dimer at the cell surface in stably transfected cells as observed with pbFRET microscopy. This technique was advantageous for probing receptors present only at the cell surface. Furthermore, these observations were also confirmed using Western blot analysis. Ligand activation of hSSTR2 resulted in the dissociation of receptor dimers to monomers as shown using both techniques. Other GPCRs have also been reported to undergo ligand-induced dissociation, including the δ-OR (29), the human cholecystokinin receptor (23), the human thyrotropin receptor (24), and the rhesus neuropeptide Y4 receptor (30). With the exception of the δ-OR, little has been shown to address the functional significance for the dissociation of receptor dimers. To address this issue for hSSTR2, we monitored the effect of blocking receptor dissociation on internalization kinetics. Using a cell-impermeable cross-linking agent to prevent receptor dissociation, we found that, in the presence of agonist, receptor internalization was impaired. Similarly, Cvejic and Devi (29) have also shown impaired receptor trafficking when δ-OR dissociation was not implicated.

Whether ligand-dependent receptor dissociation is involved in all SSTR2 species remains obscure. In a report on rat SSTR2 (37), self-associated receptors were shown by using Western blot, but agonist treatment did not have a considerable effect on receptor dimers. Interestingly, the same group also reported a SSTR2/SSTR3 heterodimer that, when treated with either a selective agonist for SSTR2 or SST-14 (agonist to both receptors), resulted in the dissociation of the heterodimeric complex and selective internalization of SSTR2 (37). A similar phenomenon was shown for the dopamine D1 and adenosine A1 receptor heterodimer, in which treatment with a D1 receptor agonist caused dissociation of the heterodimeric complex (38).

In conclusion, we show that hSSTR2 preferentially exists as a dimer at the basal state but on stimulation with agonist dissociates into monomeric receptors. Moreover, dissociation of receptor dimers is important for efficient receptor trafficking because inhibiting dimer dissociation alters internalization kinetics. These data provide a new level of understanding in the functioning of hSSTRs and may help elucidate the functional properties of other GPCRs.

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