

# Real-Time Signaling Assays Demonstrate Somatostatin Agonist Bias for Ion Channel Regulation in Somatotroph Tumor Cells

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Acromegaly is a neuroendocrine disorder caused by excess secretion of GH by somatotroph tumor cells. It is often treated with somatostatin receptor (SSTR) 2 agonists, which suppress GH secretion. SOM230 is a somatostatin analogue that targets multiple SSTRs and was recently approved for patients with treatment-resistant acromegaly. Previous reports indicate that SOM230 may function as a biased agonist, suggesting that its ability to selectively activate SSTR-dependent signaling events may contribute to its therapeutic efficacy. To better understand how SOM230 modulates Sstr2A function, which is the most commonly expressed SSTR in somatotrophs, we used real-time assays to study SOM230-dependent signaling in rat pituitary tumor cells. We observed that SOM230 suppressed cAMP production in a G $\alpha$ i-dependent manner, similar to conventional Sstr2A agonists. However, it did not cause receptor internalization as would be expected for an Sstr2A agonist. Surprisingly, SOM230 did not cause membrane hyperpolarization, which is an important mechanism by which Sstr2a activation suppresses intracellular calcium (Ca<sup>2+</sup>) accumulation and GH secretion. In fact, SOM230 inhibited the ability of conventional somatostatin analogues to control membrane potential. However, SOM230 still inhibited intracellular Ca<sup>2+</sup> accumulation in a novel, G $\beta\gamma$ -dependent manner. These studies show that SOM230 exhibits strong agonist bias in regulating signaling pathways downstream of Sstr2A that control GH secretion.

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**Freeform/Key Words:** calcium, cAMP, internalization, somatostatin, SOM230, Sstr2A

Acromegaly is a chronic hormonal disorder caused by excess GH production by a pituitary tumor [1]. The primary form of pharmacological treatment of acromegaly is to provide somatostatin receptor (SSTR) 2 agonists such as octreotide and lanreotide, which suppress GH secretion [2]. However, only two-thirds of patients respond to treatment with these compounds [3, 4]. There are five SSTR subtypes in humans, with SSTR2A and SSTR5 being the most commonly expressed in GH-secreting pituitary tumors (95% and 85% of pituitary adenomas, respectively) [5]. The lack of response to SSTR2A agonists is commonly thought to be due to inactivation of SSTR2A signaling in pituitary adenomas. Thus, the pan-SSTR agonist SOM230 (pasireotide) was developed to target other SSTRs in pituitary tumors [4, 6, 7].

Recent reports indicate that SOM230 functions as a biased agonist toward SSTRs, which may also contribute to its therapeutic efficacy. Biased agonists are ligands that can selectively activate particular signaling events downstream of a receptor [8]. In the case of

Abbreviations: Ca<sup>2+</sup>, calcium; EC<sub>50</sub>, half maximal effective concentration; ECSP, extracellular solution containing probenecid; FBS, fetal bovine serum; PTX, pertussis toxin; SSTR, somatostatin receptor; VGCC, voltage-gated calcium channel.

SOM230, it was recently shown to potently inhibit cAMP production but induce little internalization of rat Sstr2A in nonpituitary cell lines [9, 10]. This has potential therapeutic implications because SSTR2A internalization is a mechanism that is thought to down-regulate its ability to respond to ligands in humans. In this regard, it is unknown whether SOM230 behaves as a biased agonist for signaling pathways that control GH secretion.

SSTRs suppress GH secretion through multiple signaling mechanisms [11]. SSTRs can directly inhibit cAMP levels through activation of *Gai*, which then leads to a decrease in spontaneous electrical activity and inhibition of the voltage-gated calcium channels (VGCCs) that are necessary for GH secretion [12, 13]. Alternatively, Sstr2 activation can hyperpolarize the cell through cAMP-independent activation of  $K^+$  channels, which then inhibit VGCCs [12–15]. It is not known whether SOM230 exhibits biased agonism to regulate  $K^+$  channel activation or calcium ( $Ca^{2+}$ ) influx.

To better understand whether SOM230 selectively activates pathways controlling GH secretion, we compared the effects of the natural ligand SS14 with octreotide and SOM230 on the activation of cAMP-dependent and cAMP-independent signaling pathways in pituitary tumor cells. We did this using innovative real-time, live-cell assays that allow for visualization of ligand responses over an extended period. We observed that SOM230 is a cAMP-biased agonist in pituitary tumor cells, potently suppressing cAMP production yet producing little receptor internalization. We also found that, surprisingly, SOM230 did not induce membrane hyperpolarization but instead behaved as an antagonist for this pathway. However, SOM230 still partially inhibited intracellular  $Ca^{2+}$  influx, which was fractionally blocked by a G protein  $\beta\gamma$ -subunit blocking peptide. These results demonstrate that in pituitary tumor cells, SOM230 acts distinctly from other SS14 agonists to selectively inhibit intracellular  $Ca^{2+}$  accumulation without affecting membrane depolarization.

## 1. Material and Methods

### A. Reagents

Reagents were purchased from the following sources: DMEM and F12 medium from Lonza (Allendale, NJ);  $CO_2$ -independent media from Gibco/ThermoFisher (Waltham, MA); fetal bovine serum (FBS) and horse serum from Atlanta Biologicals (Flowery Branch, GA); pertussis toxin (PTX) from List Biological Laboratories Inc. (Campbell, CA); the water soluble forskolin analogue NKH477 from Cayman Chemical Company (Ann Arbor, MI); GloSensor 22F plasmid and FuGENE transfection reagent from Promega (Madison, WI); and D-Luciferin from GoldBio (St. Louis, MO). Anti-HA epitope antibody was purchased from Covance (Princeton, NJ), and goat anti-mouse secondary antibody was ordered from KPL (Gaithersburg, MD). FLIPR Membrane Potential blue dye was purchased from Molecular Devices (Sunnydale, CA). FLUO-8 AM calcium dye was purchased from TEF Labs (Austin, TX), and probenecid was obtained from Tocris (Minneapolis, MN). Geneticin (G418), poly-D-ornithine, paraformaldehyde, and BSA, Fraction V were purchased from Sigma-Aldrich (St. Louis, MO). 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt was obtained from Roche (Basel, Switzerland). Anti- $\beta\gamma$  peptide (MPS-Phosducin-like protein C-terminus) was obtained from Anaspec (Fremont, CA). Somatostatin (SS14), octreotide, and H-*p*-Chloro-Phe-D-Cys- $\beta$ -(3-pyridyl)-Ala-D-Trp-Lys-tBu-Gly-Cys-2-Nal-NH<sub>2</sub> trifluoroacetate salt (PRL-2915) were purchased from Bachem California (Torrance, CA). Signifor (pasireotide diaspertate or SOM230) was produced by Novartis Pharmaceuticals (East Hanover, NJ). The sstr1 agonist Des-AA<sup>1,2,5</sup>-[D-Trp<sup>8</sup>, IAmP<sup>9</sup>, <sup>125</sup>I-Tyr<sup>11</sup>]-CbM-SRIF (compound #27) [16], referred to as Rivier #27, was a generous gift from Jean Rivier (Salk Institute).

### B. Generation and Culture of Cell Lines

The GH4C1 rat pituitary tumor cell line was grown in F12 medium supplemented with 12.5% horse serum and 2.5% FBS. GH12C1 cells are a clone of pituitary tumor cells derived from the

rat tumor MtT/W5 that do not endogenously express SSTRs [14]. These cells were transfected with an HA3-tagged rat Sstr2A-pcDNA3 plasmid, and stable receptor-expressing clones were isolated as previously described [9]. The GH12C1-HA3-rSstr2A clone #35 (GH12C1-Sstr2A) was grown in DMEM supplemented with 10% FBS and 250  $\mu\text{g}/\text{mL}$  of G418. For membrane potential and intracellular  $\text{Ca}^{2+}$  experiments, the medium was changed, and 24 hours later the cells were seeded for experiments. Experiments were performed 16 to 18 hours after plating. Stable GH12C1 cell lines expressing a cAMP biosensor were created by transfecting the 22F GloSensor cAMP biosensor (Promega) into GH12C1-HA3-rSstr2A clone #35 cells using FuGENE (Promega) and selecting with 200  $\mu\text{g}/\text{mL}$  of hygromycin B. Clonal cell lines were isolated by limiting dilution and were screened for biosensor activity. The selected clone (GH12C1-HA3-rSstr2A-Glo clone #16, referred to as GH12C1-Sstr2A-Glo) was maintained in DMEM plus 10% FBS, 250  $\mu\text{g}/\text{mL}$  of G418, and 50  $\mu\text{g}/\text{mL}$  of hygromycin B. The HEK293-Sstr2A cell line [9] was grown in DMEM supplemented with 10% FBS.

### *C. Measurement of Real-Time Changes in cAMP Using GloSensor Assays*

Real-time, live-cell cAMP levels were measured using the cAMP GloSensor assay [17, 18]. GH12C1-Sstr2A-Glo cells were seeded at 100,000 cells per well in 96-well, white, clear-bottom, non-precoated plates (Greiner Bio-One; Monroe, NC). After 24 hours, the medium was aspirated and replaced with 90  $\mu\text{L}$  of equilibration medium (DMEM with 10% FBS, 10 mM of HEPES, 2% D-Luciferin). Plates were preincubated in a dark, humidified chamber at 28°C for 2 hours, and basal bioluminescence was measured using a PolarStar Optima multiplate reader (BMG Labtech; Cary, NC) for 10 minutes, using a 1-second integration time. Subsequently, the forskolin analogue NKH477 (10  $\mu\text{M}$ ; Cayman Chemical Company) without or with the appropriate somatostatin agonist was added in 10  $\mu\text{L}$  of equilibration buffer. Bioluminescence was measured every 2.5 minutes for 1 hour. Dose-response curves were calculated at 20 minutes after agonist addition. Each data point is an average of three different wells from a single experiment. Each experiment was repeated at least three times.

### *D. Measurement of Receptor Internalization*

Cell surface levels of the HA3-tagged Sstr2a receptor were measured using an ELISA as previously described [9]. GH12C1-Sstr2A cells were seeded at a density of 150,000 cells in 24-well plates or 100,000 cells in 96-well plates in DMEM containing 10% FBS without G418 and were cultured for 48 hours. On the day of the assay, cells were washed with PBS and equilibrated to 37°C in  $\text{CO}_2$ -independent media (Gibco/ThermoFisher). Cells were then incubated with somatostatin analogues for 30 minutes at 37°C to stimulate receptor internalization. After cells were washed with ice-cold PBS and fixed with 3% paraformaldehyde (Sigma-Aldrich) in PBS for 10 minutes, cells were blocked with 1% BSA, Fraction V (Sigma-Aldrich) in PBS for 30 minutes and then incubated overnight at 4°C with mouse anti-HA antibody (Covance). Cells were subsequently incubated with goat anti-mouse secondary antibody labeled with peroxidase (KPL), and cell surface-receptor expression was measured by incubating for 45 to 60 minutes with 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (Roche) (1 mg/mL diluted in 3.25 mM Na-perborate tetrahydrate, 40 mM citric acid monohydrate, 60 mM  $\text{NaPO}_4$  dibasic, diluted in water, pH 4.4). The optical density at 405 nm was measured with an Infinite M200 Reader (Tecan Group Ltd, Männedorf, Switzerland). For antagonist experiments, cells were pretreated with indicated concentrations of SOM230 for 5 minutes before stimulation with SS14 in the continued presence of SOM230.

### *E. Measurement of Membrane Potential*

Changes in membrane potential were measured using the FLIPR Membrane Potential blue dye kit (Molecular Devices) and a FlexStation 3 microplate reader (Molecular Devices). The

dye was reconstituted in a HEPES-buffered saline assay buffer (140 mM NaCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM KCl, 10 mM glucose, 10 mM HEPES, pH 7.4). Twenty-four hours before the experiment, cells were plated at a density of 150,000 cells per well in 96-well, black, clear-bottom plates precoated with poly-D-ornithine (Sigma-Aldrich). On the day of the assay, cells were washed with 40  $\mu$ L of HEPES-buffered saline assay buffer and then incubated for 45 minutes at 37°C with membrane potential dye at 50% to 100% of the final “in-well” dye manufacturer–recommended concentration, depending on dye lot number and cell type. The final 15-minute equilibration was done at 37°C in the prewarmed FlexStation 3. After equilibration, baseline fluorescence was measured for 30 seconds before the addition of agonists from a concentrated stock solution. Fluorescence was measured for 180 seconds with an excitation wavelength of 530 nm and an emission wavelength of 565 nm. Data were expressed as a ratio of fluorescence intensity ( $F_1/F_0$ ) calculated by dividing the signal at each time point ( $F_1$ ) by the baseline signal ( $F_0$ ) measured before the addition of agonist (control). Traces shown were calculated by subtracting control traces from agonist-treated traces [19]. Each point represents the average of three different wells. The number of replicates for each experiment is noted in the figure legends.

#### *F. Measurement of Intracellular Ca<sup>2+</sup>*

Changes in intracellular Ca<sup>2+</sup> were measured using the Ca<sup>2+</sup> indicator dye FLUO-8 AM (TefLabs) according to previously published procedures [20]. The final concentration of FLUO-8 AM was 2  $\mu$ M, and Pluronic F-127 was omitted. GH12C1-Sstr2A cells were washed with an extracellular solution containing probenecid (ECSP; 140 mM NaCl, 2 mM CaCl, 1 mM MgCl, 5 mM KCl, 10 mM glucose, 10 mM HEPES, pH 7.4, with 2 mM freshly added probenecid) and then equilibrated for 50 minutes at 37°C in an ECSP buffer containing FLUO-8 AM (2  $\mu$ M) and 0.1% BSA. After pre-equilibration, cells were washed and equilibrated with ECSP buffer for an additional 15 minutes at 37°C in a prewarmed FlexStation 3. Test compounds were added at the times shown, and changes in fluorescence were measured at an excitation wavelength of 494 nm and an emission wavelength of 535 nm. Data were expressed as described previously for membrane potential assays. Each point is an average of three different wells from a single experiment. The number of replicates for each experiment is noted in the figure legends.

#### *G. Data Analysis*

Figures show individual experiments with mean  $\pm$  SEM of three replicate wells unless otherwise specified. Experiments shown are representative of at least two independent experiments. Dose-response curves and half maximal effective concentration ( $EC_{50}$ ) values were derived using the operational fit nonlinear regression analysis, with a Hill coefficient of  $-1$  in Prism (ver. 6.0; GraphPad Software, San Diego, CA). Two-tailed  $t$  tests and one-way ANOVA with Dunnett correction were also calculated using Prism (ver. 6.0; GraphPad Software).

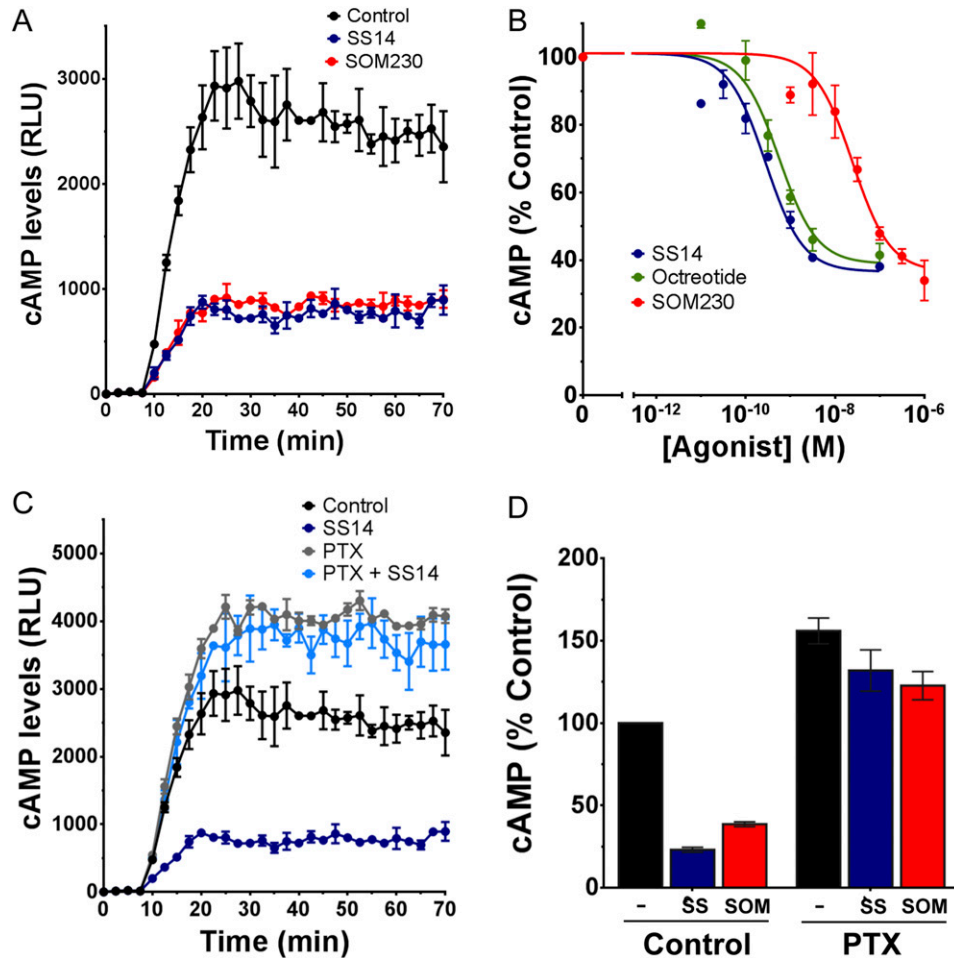
## **2. Results**

### *A. SOM230 Is a cAMP-Biased Sstr2A Ligand in Rat Pituitary Cells*

SOM230 is a somatostatin analogue that was recently approved for the treatment of acromegaly [21], but the ability of SOM230 to stimulate Sstr2A-specific signaling events that control GH secretion is not fully understood. Prior work has indicated that SOM230 functions as a biased agonist for Sstr2A, as it was unable to cause full internalization of the endogenous receptor in AR42J cells (rat exocrine pancreatic cancer cells) [10] or exogenous Sstr2A in CHO-K1 cells [9, 10]. This raised the possibility that SOM230 may exhibit bias for signaling

pathways directly relevant to suppression of GH secretion. However, the effects of SOM230 on these signaling pathways are unknown.

We first assessed the ability of SOM230 to inhibit cAMP production without causing receptor internalization in rat pituitary cells, which had not been previously tested. For these assays, we used a real-time, live-cell luminescence approach that allowed for rapid measurement of cAMP accumulation without the use of phosphodiesterase inhibitors [17, 18]. Rat pituitary GH12C1 cells expressing HA-Sstr2A were treated with the forskolin analogue NKH477, plus or minus SOM230 or somatostatin (SS14), and cAMP levels were measured. We observed that saturating concentrations of SOM230 or SS14 were equally effective at inhibiting cAMP accumulation over time (Fig. 1A).



**Figure 1.** Characterization of pituitary tumor cell cAMP responses to SOM230 using real-time, live-cell assays. GH12C1-Sstr2A-Glo cells were incubated in CO<sub>2</sub>-independent media with 2% D-Luciferin for 2 hours at 28°C. Cells were stimulated with the forskolin analogue NKH477 (10 μM) with or without varying concentrations of SS analogues, and luminescence was measured. (A) Time course of cAMP inhibitory responses for Sstr2A agonists. SS14 (100 nM); SOM230 (1 μM). cAMP response from SS14- and SOM230-treated samples were significantly different from that of control ( $P < 0.0001$ ; one-way ANOVA with Dunnett test). (B) Dose-response for cAMP inhibition by the Sstr2A agonists SS14, octreotide, and SOM230. Data were fit by nonlinear regression analysis to the operational model in GraphPad Prism, v 6.0. (C) Effect of PTX pretreatment (16 hours, 100 ng/mL) on inhibition of cAMP production by SS14 (100 nM). (D) Effect of PTX pretreatment on Sstr2A agonist cAMP response. SS14 (100 nM); SOM230 (1 μM). Data shown are mean  $\pm$  SEM from three different experiments, with three replicates per group. Two-tailed  $t$  test between control and PTX resulted in  $P < 0.0001$  for all three treatment groups. -, no ligand; RLU, relative light units; SOM, SOM230; SS, somatostatin 14.



To understand the relative potencies of each ligand, we performed dose-response experiments. SOM230, SS14, and the SS14 analogue octreotide each inhibited cAMP accumulation in a dose-dependent manner, with a rank order of potency of SS14 > octreotide > SOM230. Both SS14 analogues tested were full agonists for cAMP inhibition, as expected. The Sstr2A-specific ligand octreotide showed similar potency for cAMP inhibition as SS14 ( $EC_{50} = 0.6$  nM and  $0.2$  nM, respectively). SOM230 was less potent for cAMP inhibition, with an  $EC_{50}$  of  $58$  nM (Fig. 1B; Table 1). For each ligand, inhibition of cAMP accumulation was blocked by pretreatment with PTX ( $100$  ng/mL), indicating that these were G $\alpha$ i-mediated effects (octreotide not shown) (Fig. 1C and 1D). These results demonstrate that in pituitary tumor cells, both octreotide and SOM230 had full efficacy for cAMP inhibition and this effect was fully inhibited by PTX.

Next, we determined whether SOM230 showed a selective receptor internalization response in the pituitary tumor cell background, as previous studies showed that the extent of receptor internalization after SOM230 stimulation was dependent on the cell type examined [9, 10, 22]. We observed that both SS14 and octreotide caused maximal internalization of Sstr2A in GH12C1 cells, whereas SOM230 was completely ineffective (Fig. 2A). SS14 was more potent, with an  $EC_{50}$  threefold lower than that of octreotide ( $2.2$  nM and  $6.1$  nM, respectively) (Table 1). Because of the lack of internalization response with SOM230, we tested SOM230 activity as an antagonist for receptor internalization by comparing full dose-response curves for SS14 in the absence or presence of a 5-minute SOM230 ( $2$   $\mu$ M) pretreatment. Schild-plot analysis showed an 8.7-fold shift in SS14 potency for receptor internalization after SOM230 pretreatment (Fig. 2B). Schild-plot calculations also showed an affinity of Sstr2A for SOM230 of  $243 \pm 63$  nM. These results demonstrate that although SOM230 binds to Sstr2A, its effect on receptor internalization is minimal. Thus, SOM230 acts as a cAMP-biased agonist in pituitary tumor cells [23].

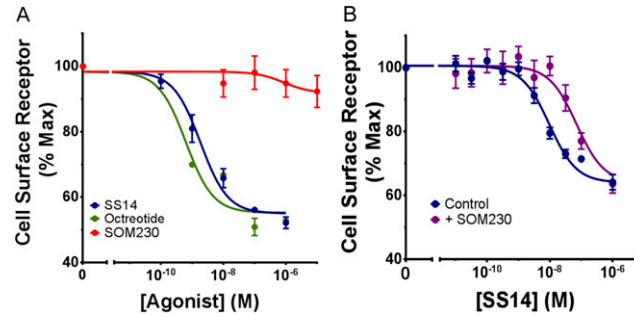
### B. SOM230 Does Not Induce Membrane Hyperpolarization and Antagonizes the Effect of SS14 on Membrane Potential

We previously showed that SS14 hyperpolarizes the cell membrane and decreases intracellular  $Ca^{2+}$  levels through a cAMP-independent mechanism to inhibit GH secretion in GH4C1 pituitary tumor cells [11]. Using a more sensitive methodology with the FLIPR Membrane Potential dye [19], we examined the effect of SS14 and octreotide on membrane potential in GH4C1 cells, which express endogenous Sstr1 and Sstr2 [14]. In accordance with previous reports [12], stimulation with SS14 ( $1$   $\mu$ M) caused a decrease in fluorescence consistent with cellular hyperpolarization, whereas the Sstr1 agonist Rivier #27 was largely ineffective. The Sstr2A-specific agonist octreotide produced a hyperpolarization response equivalent to that of SS14. Concomitant addition of octreotide and Rivier #27 had the same

**Table 1. Analogue Potencies and Maximum Effect Values for Sstr2A Signaling Pathways**

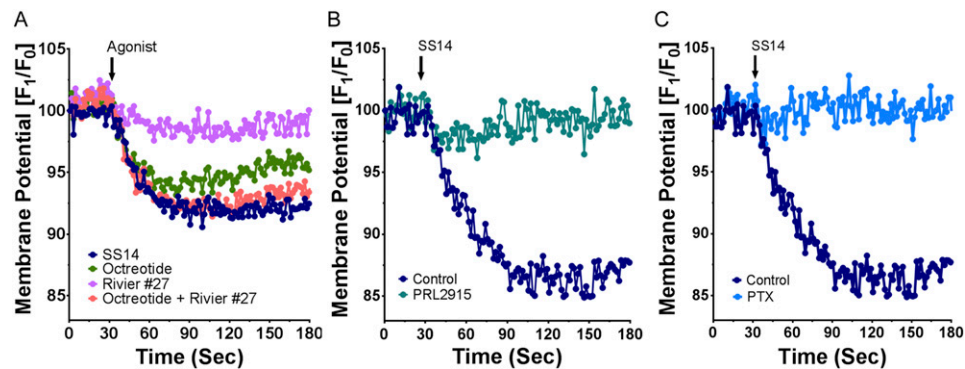
Agonist	cAMP			Internalization			Hyperpolarization			Intracellular $Ca^{2+}$	
	$EC_{50}$ (nM)	$E_{max}$	n	$EC_{50}$ (nM)	$E_{max}$	n	$EC_{50}$ (nM)	$E_{max}$	n	$E_{max}$	n
SS14	$0.2 \pm 0.02$	100%	3	$2.2 \pm 0.5$	100%	6	$3.4 \pm 1.8$	100%	4	100%	6
Octreotide	$0.6 \pm 0.1$	100%	3	$6.1 \pm 0.8$	100%	3	$0.7 \pm 0.06$	$107\% \pm 6\%$	4	$90\% \pm 6\%$	3
SOM230	$58.0 \pm 7.0$	100%	2	>10,000	$5\% \pm 3\%$	3	>10,000	$5\% \pm 4\%$	4	$46\% \pm 8\%$	6

Potencies and  $E_{max}$  values for Sstr2A-regulated signaling pathways were measured as described in Fig. 1 (cAMP inhibition), Fig. 2 (Sstr2A internalization), or Fig. 5 (membrane hyperpolarization). Potencies ( $EC_{50}$  values) were calculated using operational model nonlinear curve fitting on GraphPad Prism v 6.0. For cAMP inhibition and internalization,  $E_{max}$  was calculated from dose-response curves. For hyperpolarization and intracellular  $Ca^{2+}$ , data were first expressed as the ratio of fluorescence intensities, and then the  $E_{max}$  was calculated from this ratio using SS14 values as a normalization control.



**Figure 2.** SOM230 did not induce Sstr2A internalization in pituitary tumor cells. GH12C1-Sstr2A cells were incubated at 37°C for 30 minutes with varying concentrations of SS14 or SS analogues. Cell surface receptor was measured by ELISA and expressed as a percentage of the levels in untreated cells. (A) Cell-surface receptor levels after stimulation with varying concentrations of Sstr2A analogues (SS14, octreotide, and SOM230). Nonlinear regression analysis was performed using the operational model in GraphPad Prism, v 6.0. (B) Shift in dose-response curve for SS14 stimulation after an SOM230 pretreatment (2  $\mu$ M, 5 minutes). SOM230 (2  $\mu$ M) was kept in the wells throughout the entire experiment. Data are expressed as the mean  $\pm$  SEM from four different experiments. Each experiment contained three replicates per group. Curve fitting was performed using Gaddum/Schild-plot analysis in GraphPad Prism, v 6.0. SS, somatostatin 14.

effect as SS14 alone (104%  $\pm$  10%) (Fig. 3A). Addition of the Sstr2A-specific antagonist PRL-2915 (1  $\mu$ M) eliminated most of the hyperpolarization response produced by SS14 (1  $\mu$ M), indicating that effects on membrane potential were Sstr2A specific (Fig. 3B). The hyperpolarization effect was also abolished by PTX pretreatment (100 ng/mL), indicating a G $\alpha$ i-specific effect (Fig. 3C). These results demonstrated that somatostatin analogues induce

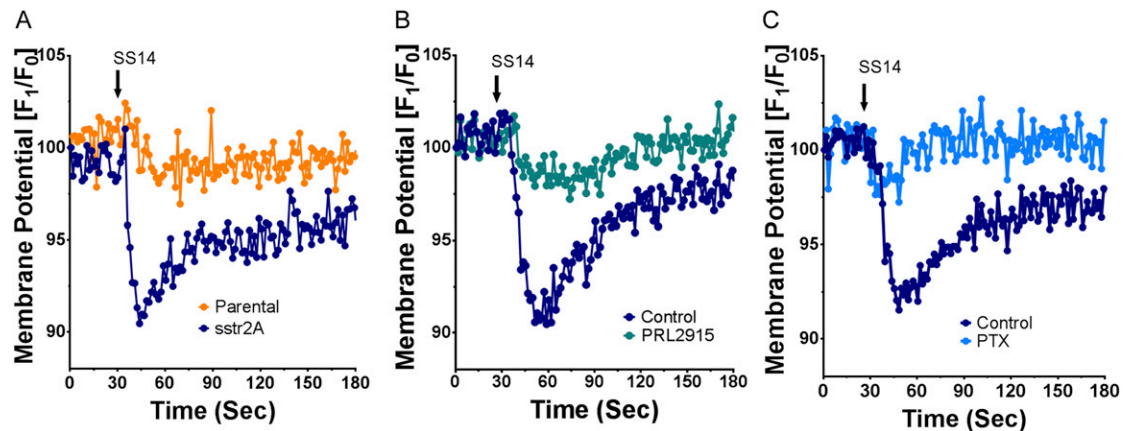


**Figure 3.** Characterization of the hyperpolarization response to Sstr2A analogues in GH4C1 cells. GH4C1 cells were incubated with FLIPR Membrane Potential dye for 30 minutes and then stimulated with SS14 or SS analogues. Changes in membrane potential were measured by fluorescence. (A) Hyperpolarization responses to SS14 (100 nM), Rivier #27 (100 nM), octreotide (100 nM), and Rivier #27 (100 nM) + Octreotide (100 nM). Hyperpolarization response from treatment groups was significantly different from that of control ( $P < 0.0001$ ; one-way ANOVA, Dunnett test). (B) Hyperpolarization response to SS14 (100 nM)  $\pm$  PRL-2915 (100 nM). Cells were pretreated with Sstr2A-specific antagonist (PRL-2915) for 15 minutes before addition of SS14. SS14-induced response was significantly different from that of PRL-2915-pretreated group ( $P < 0.0001$ ; two-tailed  $t$  test). (C) Hyperpolarization response to SS14 (100 nM) without or with PTX pretreatment (100 ng/mL, 16 to 18 hours). SS14-induced response was significantly different from that of PTX-pretreated group ( $P < 0.0001$ ; two-tailed  $t$  test). Data are expressed as the ratio of fluorescence intensity ( $F_1/F_0$ ) and are representative of (A) four or (B and C) two different experiments. Each experiment had three replicates per group. Black arrows indicate the addition of agonists 30 seconds after beginning of readout. SS, somatostatin 14.

membrane hyperpolarization and that the response is PTX sensitive. Further, it shows that in GH4C1 cells, Sstr1 activation does not induce hyperpolarization.

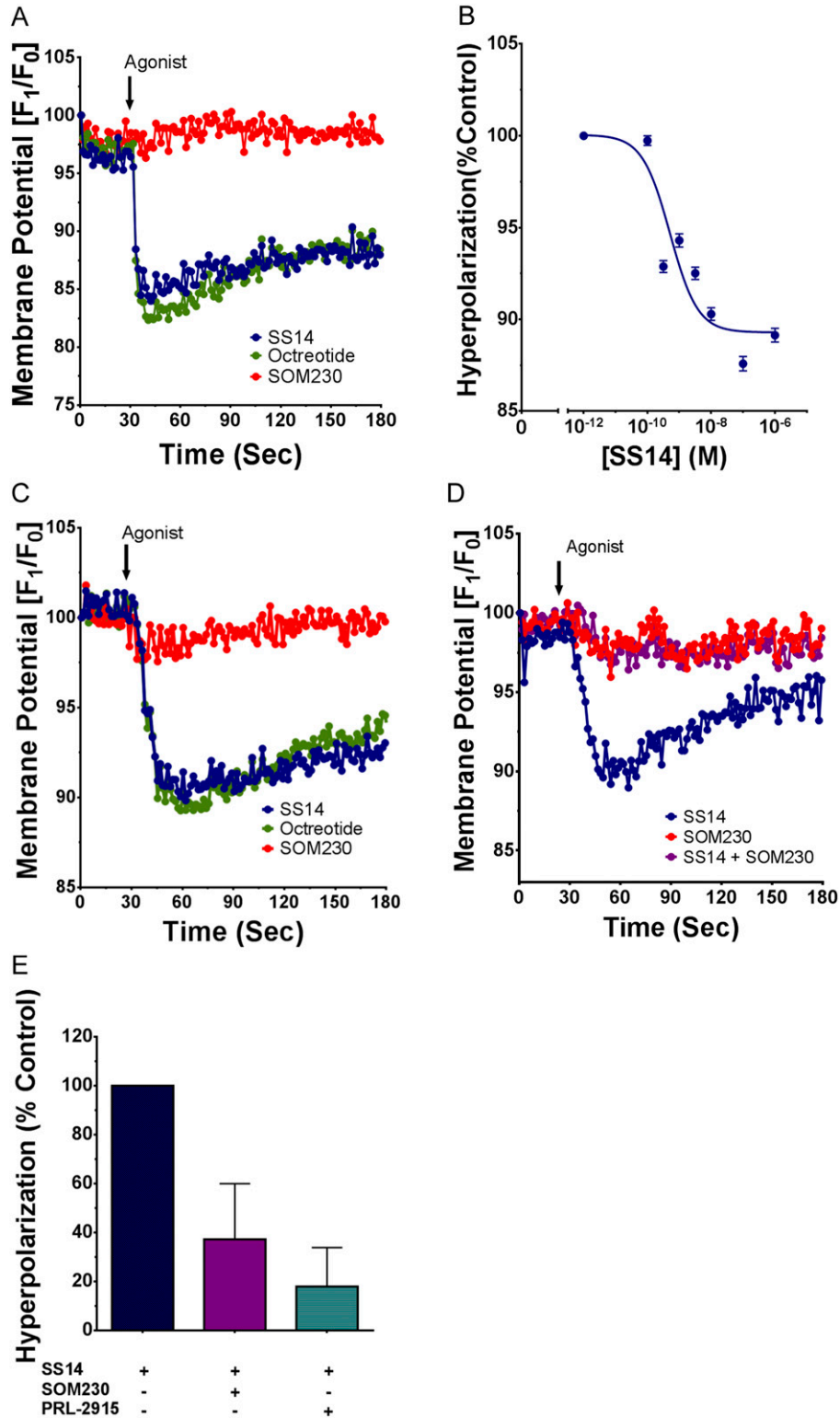
However, the presence of endogenous Sstr1 in GH4C1 cells raised the possibility of Sstr1/Sstr2A heterodimer formations and their subsequent effects upon ligand-binding affinity, receptor trafficking and desensitization, and signal transduction [24]. Therefore, we determined whether agonist effects on membrane potential could be reproduced on an Sstr2A-specific pituitary tumor cell background. For these experiments, we used GH12C1 cells, which are a clone of GH4C1 cells that do not express endogenous SSTRs, as well as GH12C1 cells stably expressing rat Sstr2A. We observed that only the cells expressing Sstr2A showed an effect on membrane potential (Fig. 4A). In this cell model, the hyperpolarization response to SS14 (1  $\mu$ M) was abolished by a 15-minute pretreatment with the Sstr2A antagonist PRL-2915 (1  $\mu$ M) (Fig. 4B) and by an overnight pretreatment with PTX (100 ng/mL) (Fig. 4C). These data support the notion that the hyperpolarization response observed in GH4C1 cells was due to Sstr2A activation.

Next, we tested whether SOM230 treatment affected membrane potential. Stimulation with SS14 caused maximal hyperpolarization, as expected, with a potency of 3.4 nM (Fig. 5A and 5B; Table 1). Octreotide also showed a maximal effect, similar to SS14 (107%  $\pm$  6%) (Fig. 5A). However, SOM230 showed no effect on membrane potential (5%  $\pm$  4%) (Fig. 5A and 5B). This surprising result was also found when GH4C1 cells, which express endogenous Sstr2A, were treated with SOM230 (Fig. 5C). Because SOM230 clearly bound to Sstr2A, we wanted to directly test whether it behaved as an antagonist for membrane hyperpolarization. Therefore, we treated GH12C1-Sstr2A cells with 2  $\mu$ M of SOM230 for 15 minutes and then stimulated with a maximal concentration of SS14 (100 nM). Cells without SOM230 pretreatment showed a hyperpolarization response, whereas the presence of SOM230 blocked SS14-induced hyperpolarization (Fig. 5D). The effect of SOM230 on cell



**Figure 4.** Sstr2A-specific stimulation with SS14 caused hyperpolarization by a PTX-sensitive mechanism. Parental GH12C1 cells or GH12C1-Sstr2A cells were incubated with FLIPR Membrane Potential dye for 30 minutes and then stimulated with SS14 (1  $\mu$ M). Changes in membrane potential were measured by fluorescence. (A) Hyperpolarization response to SS14 (1  $\mu$ M) in GH12C1-Sstr2A cells and parental GH12C1 cells, which lack endogenous Sstr2A. Responses in each cell line were significantly different ( $P < 0.001$ ; two-tailed  $t$  test). (B) Hyperpolarization response to SS14 (1  $\mu$ M) compared with SS14 (1  $\mu$ M) + PRL-2915 (1  $\mu$ M). Cells were pretreated with the Sstr2A-specific antagonist PRL-2915 at 15 minutes before the addition of SS14. Responses in PRL-2915-treated cells were significantly different from that of control ( $P < 0.0001$ ; two-tailed  $t$  test). (C) Effect of PTX pretreatment on membrane potential. Cells were stimulated with SS14 (1  $\mu$ M) with or without pretreatment with PTX (100 ng/mL, 16 to 18 hours). Response from PTX-treated cells was significantly different from that of control ( $P < 0.0001$ ; two-tailed  $t$  test). Data are expressed as the ratio of fluorescence intensity ( $F_1/F_0$ ) and are averaged from two different experiments. Each experiment had three replicates per group. Black arrows indicate the addition of agonists, 30 seconds after beginning of readout.





**Figure 5.** SOM230 stimulation of Sstr2A did not hyperpolarize pituitary tumor cells. GH12C1-Sstr2A cells were incubated with FLIPR Membrane Potential dye for 30 minutes and then stimulated with saturating concentrations of Sstr2A agonists. (A) Hyperpolarization response to SS14 (100 nM), octreotide (100 nM), and SOM230 (1  $\mu$ M). Only the SOM230-treated sample was significantly different from the SS14-treated control ( $P < 0.0001$ ; one-way ANOVA, Dunnett test). (B) Hyperpolarization dose-response to SS14 at point of maximal

effect in GH12C1-Sstr2A cells. (C) Membrane hyperpolarization in GH4C1 cells after stimulation with SS14 (100 nM), octreotide (100 nM), or SOM230 (1  $\mu$ M). Both SS14-treated and octreotide-treated samples were significantly different from SOM230-treated cells ( $P < 0.0001$ ; one-way ANOVA, Dunnett test). (D) Hyperpolarization response in GH12C1-Sstr2A cells to SS14 (100 nM), SOM230 (1  $\mu$ M), and SS14 (100 nM) with a 15-minute pretreatment of SOM230 (1  $\mu$ M). Both SOM230-treated and pretreated samples were significantly different from the SS14-treated control ( $P < 0.001$ ; one-way ANOVA, Dunnett test). (E) Maximum hyperpolarization responses in GH12C1-Sstr2A cells to SS14 (100 nM) with or without pretreatment with SOM230 (2  $\mu$ M) or the Sstr2A-specific antagonist PRL-2915 (100 nM). Data are expressed as the ratio of fluorescence intensity ( $F_1/F_0$ ). Nonlinear regression analysis was performed using the operational model in GraphPad Prism, v 6.0. Black arrows indicate the addition of agonists. Data are mean  $\pm$  SEM from three to five different experiments, three replicates per group in each experiment. +, ligand added; -, no ligand.

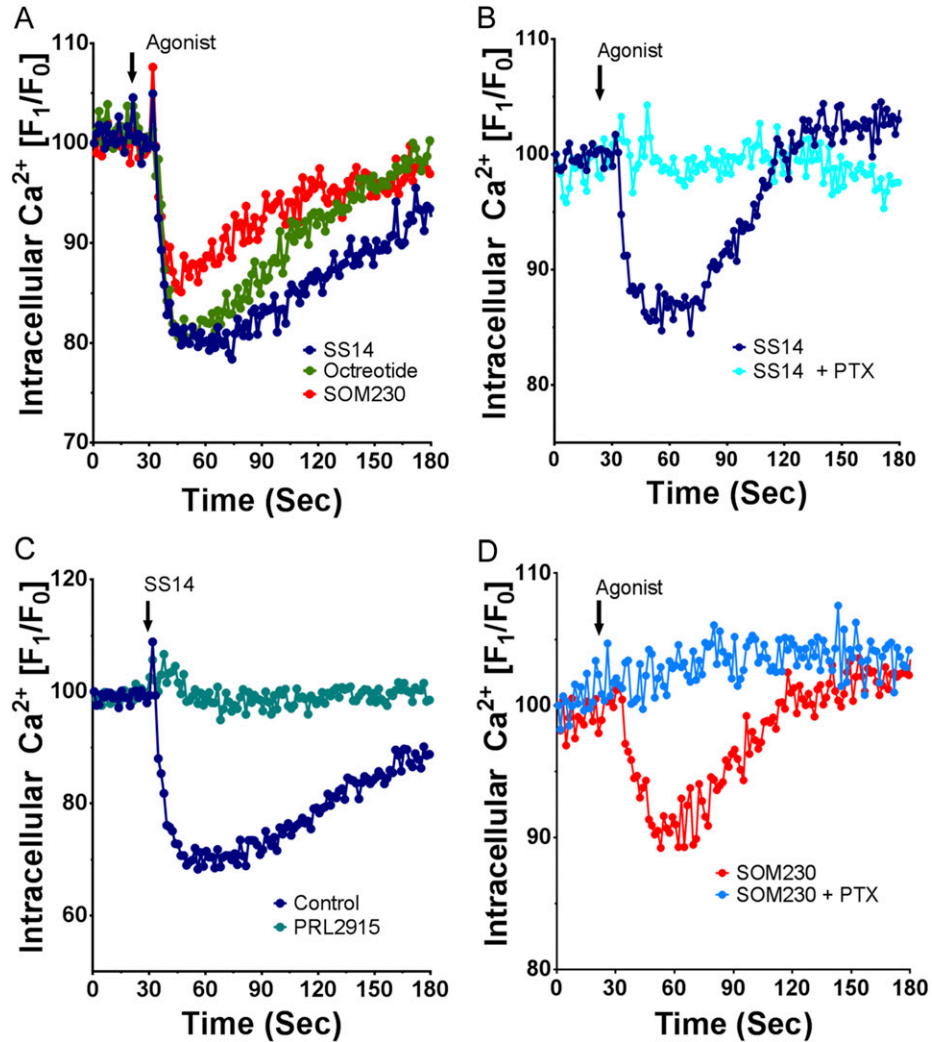
hyperpolarization was similar to the effect of the Sstr2A-specific antagonist PRL-2915 (Fig. 5E). These results demonstrate that SOM230 does not induce membrane hyperpolarization, but rather antagonizes the effect of SS14.

### C. SOM230 Inhibited Intracellular $Ca^{2+}$ Accumulation via Sstr2A

The underlying mechanism for the inhibitory effect of SS14 on prolactin and GH secretion is due to a reduction in intracellular  $Ca^{2+}$  levels within the cell [12, 13]. In GH4C1 cells, this  $Ca^{2+}$  response is mediated by L-type VGCCs [25]. Because intracellular  $Ca^{2+}$  levels directly regulate hormone secretion, we tested the effect of Sstr2A agonists on intracellular  $Ca^{2+}$  in pituitary tumor cells. We incubated GH12C1-Sstr2A cells with the fluorescent  $Ca^{2+}$ -binding dye FLOU-8-AM and then stimulated with saturating concentrations of either SS14 (100 nM), octreotide (100 nM), or SOM230 (1  $\mu$ M). We observed that stimulation with octreotide (100 nM) was nearly as effective as stimulation with SS14 in this assay ( $90\% \pm 6\%$ ) (Fig. 6A). Surprisingly, SOM230 (1  $\mu$ M) treatment also reduced intracellular  $Ca^{2+}$  levels, producing a decrease of approximately  $46\% \pm 8\%$ , compared with SS14 (Fig. 6A; Table 1). As expected, the effect of SS14 on intracellular  $Ca^{2+}$  levels in GH12C1-Sstr2A cells was PTX dependent (Fig. 6B) [25]. It was also blocked by pretreatment with the Sstr2A-specific antagonist PRL-2915 (100 nM) (Fig. 6C). Importantly, PTX (100 ng/mL) pretreatment also blocked the decrease in intracellular  $Ca^{2+}$  caused by SOM230 stimulation (1  $\mu$ M) (Fig. 6D). These results demonstrate that although SOM230 does not cause membrane depolarization, it still inhibits intracellular  $Ca^{2+}$  accumulation in pituitary tumor cells.

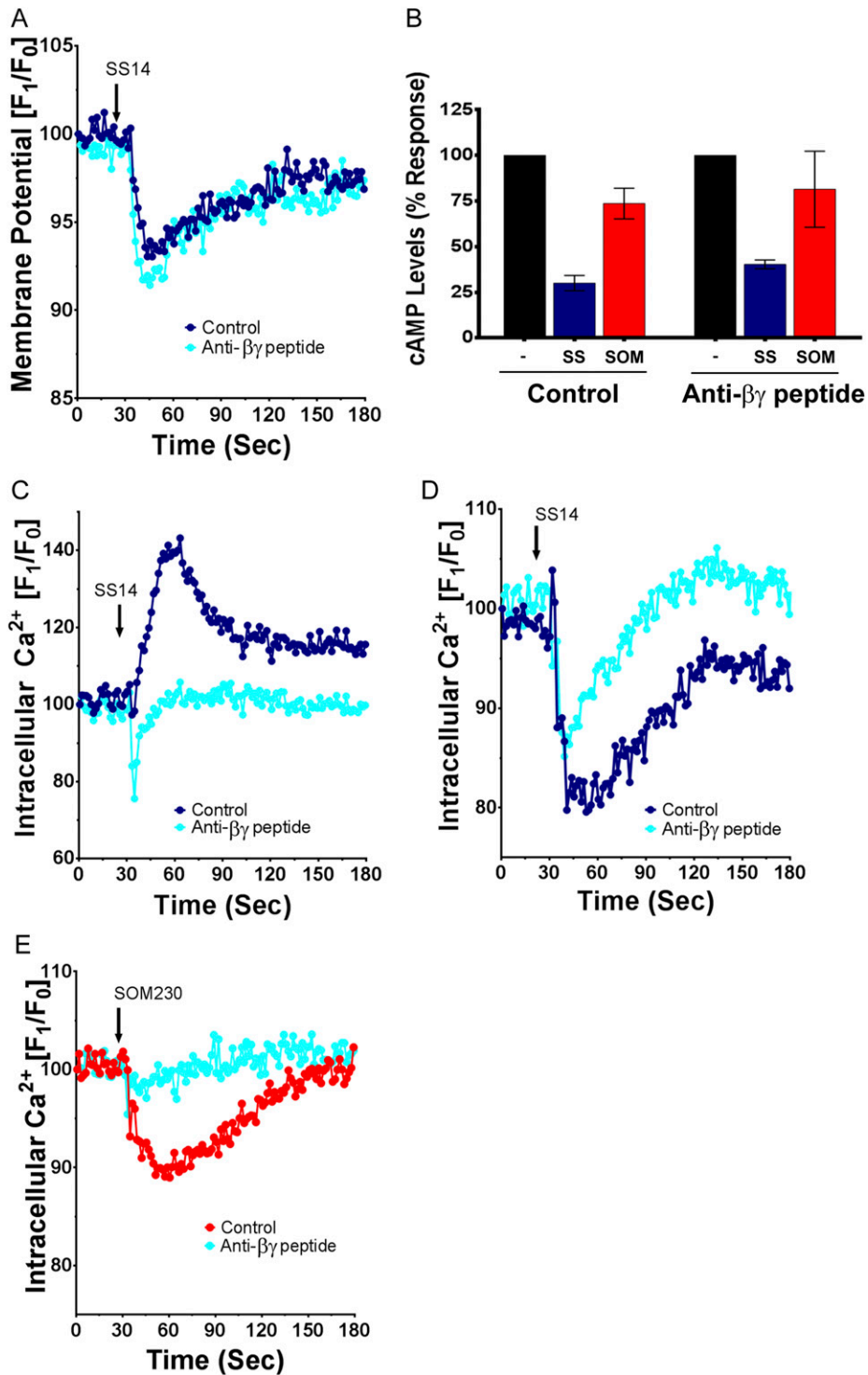
### D. G-Protein $\beta\gamma$ Subunits Partially Regulated Sstr2A Agonist-Induced Inhibition of Intracellular $Ca^{2+}$ Levels

The G protein  $\beta\gamma$  subunits are known to directly inhibit VGCCs after activation of Gai or Gao [26]. They can also directly activate inward-rectifying potassium channels, which also block VGCC activation [27]. To determine whether the G $\beta\gamma$  subunits played a role in the response to SS14 stimulation in pituitary tumor cells, we pretreated GH12C1-Sstr2A cells with anti- $\beta\gamma$  MPS-Phos, which is a membrane-permeable peptide derived from the C-terminal residues of phosducin-like protein. This peptide functions as a universal inhibitor of free G $\beta\gamma$  subunits through direct binding [28, 29]. As shown in Fig. 7A, pretreatment with MPS-Phos (1  $\mu$ M) did not inhibit SS14-stimulated membrane hyperpolarization. This was also true for the cAMP response after agonist stimulation (Fig. 7B). The lack of effect of MPS-Phos was not due to an inability to inhibit G $\beta\gamma$ , as it abolished the SS14-induced intracellular  $Ca^{2+}$  response in HEK293-Sstr2A cells (Fig. 7C) [30]. We then tested the effect of anti- $\beta\gamma$  peptide pretreatment on  $Ca^{2+}$  responses. Cells treated with SS14 (100 nM) showed a transient decrease in intracellular  $Ca^{2+}$  levels that reached steady state shortly after stimulation. Pretreatment with 1  $\mu$ M of anti- $\beta\gamma$  peptide reduced the maximal



**Figure 6.** Sstr2A agonists selectively decreased intracellular  $\text{Ca}^{2+}$  through PTX-sensitive G proteins. GH12C1-Sstr2A cells were incubated with FLUO-8-AM dye (2  $\mu\text{M}$ ) for 45 minutes, washed, and then stimulated with Sstr2A agonists. Changes in intracellular  $\text{Ca}^{2+}$  levels were measured by fluorescence. (A) Intracellular  $\text{Ca}^{2+}$  levels after stimulation with SS14 (100 nM), octreotide (100 nM), or SOM230 (1  $\mu\text{M}$ ). Both octreotide-treated and SOM230-treated samples were significantly different from SS14-control ( $P < 0.0001$ ; one-way ANOVA, Dunnett test). (B) Intracellular  $\text{Ca}^{2+}$  response to SS14 (100 nM) was blocked by PTX pretreatment (100 ng/mL, 16 to 18 hours). The effect of PTX was significant ( $P < 0.0001$ ; two-tailed  $t$  test). (C) Intracellular  $\text{Ca}^{2+}$  response to SS14 (100 nM) was blocked by pretreatment with the Sstr2A-specific antagonist PRL-2915 (100 nM) for 15 minutes, 37°C ( $P < 0.0001$ ; two-tailed  $t$  test). (D) PTX pretreatment (100 ng/mL, 16 to 18 hours) blocked intracellular  $\text{Ca}^{2+}$  response to SOM230 (1  $\mu\text{M}$ ). The effect of PTX was significant ( $P < 0.0001$ ; two-tailed  $t$  test). Data are expressed as a ratio of fluorescence intensity ( $F_1/F_0$ ). Data shown are mean  $\pm$  SEM from three independent experiments, three replicates per group per experiment. Black arrows indicate the addition of agonists 30 seconds after beginning of readout.

effect of SS14 on intracellular  $\text{Ca}^{2+}$  to approximately 70% of the control response (Fig. 7D). When cells were stimulated with SOM230 (1  $\mu\text{M}$ ), pretreatment with 1  $\mu\text{M}$  of anti- $\beta\gamma$  peptide also decreased the agonist effect on intracellular  $\text{Ca}^{2+}$  by approximately 80% (Fig. 7E). These data show that in this pituitary tumor cell model,  $G\beta\gamma$  did not directly affect Sstr2A-mediated hyperpolarization or cAMP responses but did play a role in regulating  $\text{Ca}^{2+}$  responses to both SS14 and SOM230.



**Figure 7.** Effect of anti-G $\beta\gamma$ -binding peptide on SS analogue signaling. Cells were incubated with (A) membrane potential dye, (B) cAMP GloSensor luciferin, or (C–E) intracellular  $Ca^{2+}$  dye in the presence or absence of the G protein  $\beta\gamma$  subunit peptide blocker MPS-phosducin-like protein C-terminus (MPS-Phos, 1  $\mu$ M). Cells were pretreated with MPS-Phos for 15 minutes at 28°C for cAMP GloSensor experiments or for 15 minutes at 37°C for the other experiments. (A) Hyperpolarization responses after simulation with SS14 (100 nM) or SS14 (100 nM) + MPS-Phos (1  $\mu$ M). Treatment groups were not significantly different ( $P = 0.5$ ; two-tailed  $t$  test). (B) Inhibition of NKH477 (10  $\mu$ M) stimulated cAMP production by SS14 (100 nM) or SOM230 (1  $\mu$ M) in the absence or presence of MPS-Phos (1  $\mu$ M). Response of

MPS-Phos-treated groups was not significantly different from that of nontreated groups (two-tailed *t* test). (C) HEK293-Sstr2A cells were incubated with the FLUO-8<sub>AM</sub> dye for 45 minutes, washed, and then incubated with the anti-βγ peptide MPS-Phos (2 μM) for 15 minutes. Cells were then treated with saturating concentrations of SS14 (100 nM). The effect of MPS-Phos was significant (*P* < 0.0001; two-tailed *t* test). Data are mean ± SEM from two different experiments, three replicates per group per experiment. (D) Intracellular Ca<sup>2+</sup> levels after stimulation with SS14 (100 nM) or SS14 (100 nM) + MPS-Phos (1 μM). The effect of MPS-Phos was significant (*P* < 0.0001; two-tailed *t* test). (E) Intracellular Ca<sup>2+</sup> levels after stimulation with SOM230 (1 μM) or SOM230 (1 μM) + MPS-Phos (1 μM). The effect of MPS-Phos was significant (*P* < 0.0001; two-tailed *t* test). Data are expressed as a ratio of fluorescence intensity (F<sub>1</sub>/F<sub>0</sub>). (A, B, D, and E) Data show the mean ± SEM from three independent experiments, three replicates per group per experiment. Black arrows indicate the addition of agonists 30 seconds after the start of the experiment. -, no ligand; SOM, SOM230; SS, somatostatin 14.

### 3. Discussion

In the current study, we used real-time, live-cell assays to demonstrate that SOM230 exhibited agonist bias in regulating signaling pathways downstream of SSTR2A. These pathways, both cAMP dependent and cAMP independent, ultimately inhibited secretion of GH [12]. SOM230 functional selectivity at Sstr2A was previously demonstrated in non-pituitary cell lines, where SOM230 potently inhibited cAMP production but had no effect on receptor internalization [9, 10]. However, acromegaly is a disease of the pituitary, and we sought to confirm whether SOM230 behaved similarly in a pituitary tumor cell background [14]. Our results showed that SOM230 was indeed biased toward cAMP inhibition over receptor internalization in pituitary tumor cells. Although the potencies observed in these cells were lower than with nonpituitary tumor models, the rank order of potency for all compounds tested remained the same. Using pretreatment with PTX, we were also able to confirm that the Sstr2A response in pituitary tumor cells is mediated through the Gai/o heterotrimeric G proteins.

SSTRs have been shown to inhibit GH secretion through two distinct signaling mechanisms. Somatostatin agonists can directly inhibit cAMP levels within the cell through activation of Gai [12, 13]. This leads to a decrease of GH release in pituitary tumors through modulation of ion channel activities, including the hyperpolarization-activated and cyclic nucleotide-regulated channels, which ultimately leads to a decrease in spontaneous electrical activity and inhibition of VGCCs [31]. An alternative mechanism, which is cAMP independent, involves direct activation of plasma membrane potassium channels, which causes cellular hyperpolarization. This in turn deactivates VGCCs, thereby preventing an increase in intracellular Ca<sup>2+</sup> concentrations normally required for GH release [25, 31, 32]. Our results show that unlike SS14 or octreotide, SOM230 stimulated the Sstr2A receptor but did not hyperpolarize the cell, thus demonstrating agonist bias for downstream signaling. It is noteworthy that this signaling bias may not be conserved among Sstr subtypes, as the mouse pituitary cell line AtT-20 showed a strong Sstr5-mediated hyperpolarization response to SOM230 [33]. Because SOM230 did not stimulate membrane hyperpolarization in Sstr2A-expressing cells, we were surprised to observe that it still partially decreased intracellular Ca<sup>2+</sup> levels in a Gβγ-dependent manner. This may be due to the fact that the activities of VGCCs have been regulated by interaction with Gβγ [12, 26, 34, 35].

In summary, we have demonstrated that SOM230 behaves as a biased agonist of Sstr2A in rat pituitary tumor cells. Namely, it inhibited cAMP production but failed to induce significant receptor internalization. We have also shown that SOM230 did not stimulate cellular hyperpolarization. However, it still partially reduced intracellular Ca<sup>2+</sup> levels, indicating that it preserves both putative mechanisms for inhibiting GH secretion in pituitary tumors. Because SOM230 did not induce Sstr2A internalization, it exhibited a signaling bias that would, in theory, be desirable for treating GH-secreting pituitary tumors. In the future, it will be interesting to assess whether other Sstr2A-biased agonists being considered as



therapeutic options for treatment-resistant acromegaly conserve these desirable features. It will also be important to assess the relative importance of cAMP-dependent and cAMP-independent signaling pathways in controlling GH secretion.

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**Disclosure Summary:** The authors have nothing to disclose.

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## References and Notes

1. Öberg K, Lamberts SW. Somatostatin analogues in acromegaly and gastroenteropancreatic neuroendocrine tumours: past, present and future. *Endocr Relat Cancer*. 2016;**23**(12):R551–R566.
2. McKeage K. Pasireotide in acromegaly: a review. *Drugs*. 2015;**75**(9):1039–1048.
3. Hofland LJ, van der Hoek J, van Koetsveld PM, de Herder WW, Waaijers M, Sprij-Mooij D, Bruns C, Weckbecker G, Feelders R, van der Lely AJ, Beckers A, Lamberts SW. The novel somatostatin analog SOM230 is a potent inhibitor of hormone release by growth hormone- and prolactin-secreting pituitary adenomas in vitro. *J Clin Endocrinol Metab*. 2004;**89**(4):1577–1585.
4. Schmid HA. Pasireotide (SOM230): development, mechanism of action and potential applications. *Mol Cell Endocrinol*. 2008;**286**(1-2):69–74.
5. Colao A, Auriemma RS, Lombardi G, Pivonello R. Resistance to somatostatin analogs in acromegaly. *Endocr Rev*. 2011;**32**(2):247–271.
6. van der Hoek J, de Herder WW, Feelders RA, van der Lely AJ, Uitterlinden P, Boerlin V, Bruns C, Poon KW, Lewis I, Weckbecker G, Krahnke T, Hofland LJ, Lamberts SW. A single-dose comparison of the acute effects between the new somatostatin analog SOM230 and octreotide in acromegalic patients. *J Clin Endocrinol Metab*. 2004;**89**(2):638–645.
7. Petersenn S, Schopohl J, Barkan A, Mohideen P, Colao A, Abs R, Buchelt A, Ho YY, Hu K, Farrall AJ, Melmed S, Biller BM; Pasireotide Acromegaly Study Group. Pasireotide (SOM230) demonstrates efficacy and safety in patients with acromegaly: a randomized, multicenter, phase II trial. *J Clin Endocrinol Metab*. 2010;**95**(6):2781–2789.
8. Kenakin T, Christopoulos A. Signalling bias in new drug discovery: detection, quantification and therapeutic impact. *Nat Rev Drug Discov*. 2013;**12**(3):205–216.
9. Liu Q, Cescato R, Dewi DA, Rivier J, Reubi JC, Schonbrunn A. Receptor signaling and endocytosis are differentially regulated by somatostatin analogs. *Mol Pharmacol*. 2005;**68**(1):90–101.
10. Kao YJ, Ghosh M, Schonbrunn A. Ligand-dependent mechanisms of sst2A receptor trafficking: role of site-specific phosphorylation and receptor activation in the actions of biased somatostatin agonists. *Mol Endocrinol*. 2011;**25**(6):1040–1054.
11. Koch BD, Dorflinger LJ, Schonbrunn A. Pertussis toxin blocks both cyclic AMP-mediated and cyclic AMP-independent actions of somatostatin: evidence for coupling of Ni to decreases in intracellular free calcium. *J Biol Chem*. 1985;**260**(24):13138–13145.
12. Koch BD, Blalock JB, Schonbrunn A. Characterization of the cyclic AMP-independent actions of somatostatin in GH cells: I. an increase in potassium conductance is responsible for both the hyperpolarization and the decrease in intracellular free calcium produced by somatostatin. *J Biol Chem*. 1988;**263**(1):216–225.
13. Holl RW, Thorner MO, Leong DA. Intracellular calcium concentration and growth hormone secretion in individual somatotropes: effects of growth hormone-releasing factor and somatostatin. *Endocrinology*. 1988;**122**(6):2927–2932.
14. Schonbrunn A, Tashjian H Jr. Characterization of functional receptors for somatostatin in rat pituitary cells in culture. *J Biol Chem*. 1978;**253**(18):6473–6483.
15. Jian K, Barhoumi R, Ko ML, Ko GY. Inhibitory effect of somatostatin-14 on L-type voltage-gated calcium channels in cultured cone photoreceptors requires intracellular calcium. *J Neurophysiol*. 2009;**102**(3):1801–1810.

16. Rivier JE, Hoeger C, Erchegyi J, Gulyas J, DeBoard R, Craig AG, Koerber SC, Wenger S, Waser B, Schaer JC, Reubi JC. Potent somatostatin undecapeptide agonists selective for somatostatin receptor 1 (sst1). *J Med Chem*. 2001;**44**(13):2238–2246.
17. Binkowski BF, Butler BL, Stecha PF, Eggers CT, Otto P, Zimmerman K, Vidugiris G, Wood MG, Encell LP, Fan F, Wood KV. A luminescent biosensor with increased dynamic range for intracellular cAMP. *ACS Chem Biol*. 2011;**6**(11):1193–1197.
18. Felouzis V, Hermand P, de Laissardière GT, Combadière C, Deterre P. Comprehensive analysis of chemokine-induced cAMP-inhibitory responses using a real-time luminescent biosensor. *Cell Signal*. 2016;**28**(1):120–129.
19. Knapman A, Connor M. Fluorescence-based, high-throughput assays for  $\mu$ -opioid receptor activation using a membrane potential-sensitive dye. *Methods Mol Biol*. 2015;**1230**:177–185.
20. Luo J, Zhu Y, Zhu MX, Hu H. Cell-based calcium assay for medium to high throughput screening of TRP channel functions using FlexStation 3. *J Vis Res*. 2011;**54**:pii–3149.
21. Gadelha MR, Bronstein MD, Brue T, Coculescu M, Fleseriu M, Guitelman M, Pronin V, Raverot G, Shimon I, Lievre KK, Fleck J, Aout M, Pedroncelli AM, Colao A; Pasireotide C2402 Study Group. Pasireotide versus continued treatment with octreotide or lanreotide in patients with inadequately controlled acromegaly (PAOLA): a randomised, phase 3 trial. *Lancet Diabetes Endocrinol*. 2014;**2**(11):875–884.
22. Lesche S, Lehmann D, Nagel F, Schmid HA, Schulz S. Differential effects of octreotide and pasireotide on somatostatin receptor internalization and trafficking in vitro. *J Clin Endocrinol Metab*. 2009;**94**(2):654–661.
23. Stahl EL, Zhou L, Ehlert FJ, Bohn LM. A novel method for analyzing extremely biased agonism at G protein-coupled receptors. *Mol Pharmacol*. 2015;**87**(5):866–877.
24. Chalabi M, Duluc C, Caron P, Vezzosi D, Guillermet-Guibert J, Pyronnet S, Bousquet C. Somatostatin analogs: does pharmacology impact antitumor efficacy? *Trends Endocrinol Metab*. 2014;**25**(3):115–127.
25. Koch BD, Schonbrunn A. Characterization of the cyclic AMP-independent actions of somatostatin in GH cells: II. an increase in potassium conductance initiates somatostatin-induced inhibition of prolactin secretion. *J Biol Chem*. 1988;**263**(1):226–234.
26. Herlitze S, Garcia DE, Mackie K, Hille B, Scheuer T, Catterall WA. Modulation of Ca<sup>2+</sup> channels by G-protein  $\beta\gamma$  subunits. *Nature*. 1996;**380**(6571):258–262.
27. Altier C. GPCR and voltage-gated calcium channels (VGCC) signaling complexes. *Subcell Biochem*. 2012;**63**:241–262.
28. Chang M, Zhang L, Tam JP, Sanders-Bush E. Dissecting G protein-coupled receptor signaling pathways with membrane-permeable blocking peptides: endogenous 5-HT<sub>2C</sub> receptors in choroid plexus epithelial cells. *J Biol Chem*. 2000;**275**(10):7021–7029.
29. Morrey C, Estephan R, Abbott GW, Levi R. Cardioprotective effect of histamine H<sub>3</sub>-receptor activation: pivotal role of G  $\beta\gamma$ -dependent inhibition of voltage-operated Ca<sup>2+</sup> channels. *J Pharmacol Exp Ther*. 2008;**326**(3):871–878.
30. Cescato R, Loesch KA, Waser B, Mäcke HR, Rivier JE, Reubi JC, Schonbrunn A. Agonist-biased signaling at the sst2A receptor: the multi-somatostatin analogs KE108 and SOM230 activate and antagonize distinct signaling pathways. *Mol Endocrinol*. 2010;**24**(1):240–249.
31. Stojilkovic SS. Molecular mechanisms of pituitary endocrine cell calcium handling. *Cell Calcium*. 2012;**51**(3-4):212–221.
32. Stojilkovic SS, Tabak J, Bertram R. Ion channels and signaling in the pituitary gland. *Endocr Rev*. 2010;**31**(6):845–915.
33. Günther T, Culler M, Schulz S. Research resource: real-time analysis of somatostatin and dopamine receptor signaling in pituitary cells using a fluorescence-based membrane potential assay. *Mol Endocrinol*. 2016;**30**(4):479–490.
34. Tallent M, Liapakis G, O'Carroll AM, Lolait SJ, Dichter M, Reisine T. Somatostatin receptor subtypes SSTR2 and SSTR5 couple negatively to an L-type Ca<sup>2+</sup> current in the pituitary cell line AtT-20. *Neuroscience*. 1996;**71**(4):1073–1081.
35. Dolphin AC. G protein modulation of voltage-gated calcium channels. *Pharmacol Rev*. 2003;**55**(4):607–627.