

# Comparison of functional profiles at human recombinant somatostatin sst<sub>2</sub> receptor: simultaneous determination of intracellular Ca<sup>2+</sup> and luciferase expression in CHO-K1 cells

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**1** Somatostatin (somatotropin release inhibiting factor; SRIF) acts *via* five G protein-coupled receptors (sst<sub>1</sub>–sst<sub>5</sub>) that modulate multiple cellular effectors. The aim of this study was to compare two functional effects of the human sst<sub>2</sub> receptor stably expressed in CHO-K1 cells in a single experiment using a duplex assay for intracellular calcium and serum response element (SRE)-driven luciferase expression.

**2** Intracellular calcium was measured using a fluorometric imaging plate reader II (FLIPR II). SRIF-14 rapidly and transiently increased intracellular calcium with a pEC<sub>50</sub> of 8.74 ± 0.03 (*n* = 52). At 5 h after FLIPR II measurements, luciferase expression was determined. SRIF-14 concentration-dependently increased luciferase expression (pEC<sub>50</sub> = 9.06 ± 0.03, *n* = 52).

**3** Natural and synthetic agonist/antagonist ligands for SRIF receptors were tested in the duplex assay. Correlation of agonist potencies and efficacies between the two responses were significant (*r*<sup>2</sup> = 0.83 and 0.90, pEC<sub>50</sub> and *E*<sub>max</sub>, respectively).

**4** Pertussis toxin pretreatment reduced SRIF-14/octreotide-mediated intracellular calcium increases by 45–47% and luciferase expression by 95–98%.

**5** Thapsigargin pretreatment abolished the SRIF-14/octreotide-mediated intracellular calcium increase but had no effect on luciferase expression.

**6** In conclusion, SRIF stimulates an increase in intracellular calcium and SRE-luciferase expression *via* human sst<sub>2</sub> receptors in CHO-K1 cells. The increase in luciferase is mediated *via* G<sub>i</sub>/G<sub>o</sub> while intracellular calcium increase is mediated by both G<sub>i</sub>/G<sub>o</sub> proteins and pertussis toxin-insensitive G proteins, and is mainly *via* release of calcium from intracellular stores. SRIF ligands display a similar recognition profile suggesting that the ligand/receptor/G protein/effector interaction is similar for the two parameters.

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**Keywords:** Somatostatin receptor subtype 2; CHO-K1 cells; FLIPR II; SRE-luciferase; duplex assay; pertussis toxin; thapsigargin

**Abbreviations:** CHO-K1, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's Medium; FLIPR II, fluorometric imaging plate reader II; FLU, fluorescence light units; GppNHp, 5'-guanylyl-imidodiphosphate; MAP kinase, mitogen-activated protein kinase; RLU, relative luminescence units; SRE, serum response element; SRF, serum response factor; SRIF, somatostatin/somatotropin release inhibiting factor; sst, somatostatin receptor; TCF, ternary complex factor

## Introduction

The neuropeptide somatostatin (somatotropin release inhibiting factor; SRIF) acts *via* five G protein-coupled receptors

(sst<sub>1</sub>–sst<sub>5</sub>; Hoyer *et al.*, 1995). According to structural and pharmacological similarities, the five receptors can be classified in two groups – SRIF<sub>1</sub> which consists of sst<sub>2</sub>, sst<sub>3</sub> and sst<sub>5</sub>, and SRIF<sub>2</sub> which consists of sst<sub>1</sub> and sst<sub>4</sub> (Hoyer *et al.*, 1995; Hannon *et al.*, 2002).

All five receptors are coupled to adenylyl cyclase *via* G<sub>i</sub>/G<sub>o</sub> proteins, leading to inhibition of cyclic AMP (cAMP) accumulation (Patel *et al.*, 1994; Carruthers *et al.*, 1999; Siehler & Hoyer, 1999b). In addition, the receptors have been shown to modulate multiple cellular effectors including phospholipase C, Ca<sup>2+</sup> channels, K<sup>+</sup> channels, mitogen-activated protein kinases (MAP kinases) and phosphotyrosine phosphatases (Patel & Srikant, 1997; Meyerhof, 1998).

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However, it becomes apparent when reviewing the literature, on the functional coupling of SRIF receptors and the mechanisms involved, that there are many discrepancies. Thus, while one group may find that one receptor subtype activates an effector pathway, another group using a different system may find that the same receptor has no effect, or even the opposite effect, on the same pathway. For example, regarding phospholipase C modulation, some studies have shown that all five recombinant receptors are able to stimulate phospholipase C (Akbar *et al.*, 1994; Tomura *et al.*, 1994; Komatsuzaki *et al.*, 1997), whereas other studies have shown that some receptor subtypes are unable to stimulate phospholipase C (Bito *et al.*, 1994). In native systems, the situation is even more complicated and SRIF can both stimulate and inhibit phospholipase C depending on the system examined (Malm *et al.*, 1991; Lachowicz *et al.*, 1992; 1994; Kamiya *et al.*, 1993; Murthy *et al.*, 1996; Siehler & Hoyer, 1999c; Romoser *et al.*, 2001). This is undoubtedly due to differences in the cytosolic composition between different native systems and between native and recombinant systems, as well as between assay conditions (e.g. whole cells vs membranes) and agonists used. It appears as though the effects of a given receptor at a given signalling pathway are dependent on many factors including cell type, the G protein subtypes present and the ligand used, and whether or not this really reflects the physiological situation is difficult to understand.

In our laboratory it has been shown that the efficacy profile of SRIF ligands at the same receptor subtype may vary according to the second messenger studied, and, that in radioligand binding studies, the affinity profile varied according to the radioligand used. This suggests that (i) multiple agonist-specific receptor conformations can be achieved at recombinant SRIF receptor subtypes, with various binding sensitivity to GTP, (ii) the nature of agonist-modulated receptor/G protein/effector interactions might be more complex than initially suggested by the ternary complex model and (iii) different rank orders of apparent potency can be observed at SRIF receptors depending on the nature of the pharmacological method used (radioligand binding, [ $^3S$ ]GTP $\gamma$ S binding, adenylyl cyclase activity, phospholipase C activity). These data suggest that ligand-selective receptor conformations may be occurring, as proposed for the human 5-HT $_{2c}$  receptor (Berg *et al.*, 1998a–c; Leff & Scaramellini, 1998; Scaramellini & Leff, 1998; Siehler & Hoyer, 1999a, b; Siehler *et al.*, 1999).

The aim of this study was to create a duplex assay in which it would be possible to examine the effects of different agonists at SRIF receptors by measuring two functional parameters in a single experiment, thus enabling direct comparison of ligand-induced effects in intact cells without complications due to varying assay conditions. This was carried out by examining the effects of SRIF, *via* human  $ss_2$  stably expressed in Chinese hamster ovary (CHO-K1) cells, on intracellular calcium and serum response element (SRE)-driven luciferase expression.

SRIF has previously been shown to increase intracellular  $Ca^{2+}$  levels *via* both recombinant and native receptors (Marin *et al.*, 1991; Akbar *et al.*, 1994; Tomura *et al.*, 1994; Chen & Tashjian, 1994; Taylor, 1995; Chen *et al.*, 1997; Siehler & Hoyer, 1999c; Akopian *et al.*, 2000; Romoser *et al.*, 2001; Roskopf *et al.*, 2003), and in this study intracellular  $Ca^{2+}$  changes were determined *via* fluorescence changes using a fluorometric imaging plate reader II (FLIPR II).

Determination of luciferase expression was possible as a marker of functional receptor activation since the CHO-K1 cells used to express the  $ss_2$  receptor were also stably transfected with the luciferase reporter gene under the control of the promoter sequence SRE. The SRE is regulated by transcription factors that are constitutively bound to DNA and are rapidly phosphorylated and activated in response to many extracellular signals including ligands which act at G protein-coupled receptors (Hill & Treisman, 1995; Treisman, 1995; Chai & Tarnawski, 2002). SRIF receptors have been shown in our lab to concentration-dependently increase luciferase expression *via* the SRE, providing a sensitive marker of agonist efficacy that differentiates between full and partial agonists, and antagonists (Feuerbach *et al.*, 2000; Nunn *et al.*, 2002; 2003a–c).

## Methods

### Cell culture

CHO-K1 cells, stably expressing the recombinant SRE-luciferase reporter gene, and human SRIF  $ss_2$  receptors at levels which can be considered to be close to physiological ( $120\text{ fmol mg}^{-1}$  protein compared to  $105\text{ fmol mg}^{-1}$  protein in rat cortex; Schoeffer *et al.*, 1995), were cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 nutrient mix (1:1 + glutamax I and pyridoxine), supplemented with 10% ( $v/v$ ) foetal bovine serum at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ , 95% relative humidity. Low concentrations of selection antibiotics were also included in the culture medium (geneticin sulphate (G418),  $100\text{ }\mu\text{g ml}^{-1}$  and hygromycin B,  $100\text{ }\mu\text{g ml}^{-1}$ ). The cells were passaged every 2 days by washing with phosphate-buffered saline and brief incubation with trypsin ( $0.5\text{ mg ml}^{-1}$ )/EDTA ( $0.2\text{ mg ml}^{-1}$ ). For storage, the cells were resuspended in medium containing 10% ( $v/v$ ) dimethyl sulphoxide and 20% ( $v/v$ ) foetal bovine serum, and frozen in liquid nitrogen.

### Radioligand binding assay

For crude cell membrane preparations, cells were washed with HEPES (10 mM, pH 7.5), scraped off the culture plates with the same buffer, and centrifuged at  $4^\circ\text{C}$  for 5 min at  $2500 \times g$ . The cell pellet was either stored at  $-80^\circ\text{C}$  or used directly. Before the experiments, cell membranes were resuspended in binding assay buffer (10 mM HEPES, 0.5% ( $w/v$ ) bovine serum albumin, pH 7.5) by homogenisation with a Polytron homogeniser at 50 Hz for 20 s.

Cell homogenate ( $150\text{ }\mu\text{l}$ ) was incubated with  $50\text{ }\mu\text{l}$  of radioligand ( $2175\text{ Ci mmol}^{-1}$ , 25–35 pM final concentration), in binding assay buffer containing 5 mM  $\text{MgCl}_2$ , and the protease inhibitor bacitracin ( $5\text{ }\mu\text{g ml}^{-1}$ ), and either  $50\text{ }\mu\text{l}$  assay buffer (total binding); SRIF-14 ( $10\text{ }\mu\text{M}$ ; nonspecific binding); or various test compound concentrations. Experiments were conducted in triplicate. Incubation was terminated after 1 h at room temperature by vacuum filtration through glass fibre filters presoaked in 0.3% ( $v/v$ ) polyethyleneimine. The filters were washed three times with ice-cold Tris-HCl buffer (10 mM) containing 154 mM NaCl, pH 7.4, and dried. Bound radioactivity was determined in a  $\beta$  scintillation counter (Packard TopCount) using scintillation liquid (80% counting efficiency).

### Codetermination of intracellular $\text{Ca}^{2+}$ and luciferase expression

Cells were seeded at 9000 cells  $\text{well}^{-1}$  in 384-well black-walled clear bottom, poly-D-lysine coated plates. After 24 h, the medium was removed and cells were washed once with phosphate-buffered saline and serum-deprived overnight in assay buffer (130 mM NaCl, 5.4 mM KCl, 1.8 mM  $\text{CaCl}_2$ , 0.8 mM  $\text{MgSO}_4$ , 0.9 mM  $\text{NaH}_2\text{PO}_4$ , 25 mM glucose, 20 mM HEPES, pH 7.4) containing bovine serum albumin (1%  $\text{w v}^{-1}$ ). Where pertussis toxin was used it was included in this buffer (100 ng  $\text{ml}^{-1}$ ).

On the day of the experiment, the cells were treated with assay buffer containing the  $\text{Ca}^{2+}$ -sensitive fluorescent dye Fluo4-AM (2  $\mu\text{M}$ ), and probenecid (0.1 mM). Where thapsigargin was used, it was included in this buffer (1  $\mu\text{M}$ ). After 1 h, plates were washed twice with, and resuspended in, assay buffer containing probenecid (0.1 mM) using a multiplate washer. In experiments to determine the effect of extracellular  $\text{Ca}^{2+}$ , buffer without  $\text{Ca}^{2+}$  and containing EGTA (1 mM) was used to wash and refill the plates after the loading step. The plates were placed into a FLIPR II (Molecular Devices, Sunnyvale, CA, U.S.A.) and baseline fluorescence (fluorescence light units, FLU) was measured (five measurements, 2 s each; laser excitation 488 nm at 0.6–1 W, CCD camera exposure 0.4 s) before addition of buffer alone (basal) or containing test compound. Fluorescence measurements were then continued every 1 s for 120 s followed by every 4 s for 240 s.

After a further 5 h incubation (37°C, 5%  $\text{CO}_2$ , 95% relative humidity), the buffer containing test compounds was removed and cells were lysed in 10  $\mu\text{l}$  lysis buffer (25 mM Tris-phosphate, 2 mM DL-dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid, 10% ( $\text{v v}^{-1}$ ) glycerol, 1% ( $\text{v v}^{-1}$ ) triton-X, pH 7.8). A volume of 20  $\mu\text{l}$  luciferase assay buffer (20 mM Tris, 1.07 mM  $\text{Mg}(\text{CO}_3)_4$ , 2.67 mM  $\text{MgSO}_4$ , 0.1 mM EDTA, 33.3 mM DL-dithiothreitol, 0.27 mM coenzyme A, 0.47 mM D-luciferin, 0.53 mM ATP, pH 7.8) was added to each well and luciferase expression was determined by measuring emitted luminescence (relative luminescence units: RLU) using a Viewlux<sup>TM</sup> ultraHTS microplate imager (Perkin Elmer Life Sciences; exposure time 30 s).

### Cell growth assay

A total of 500,000 cells  $\text{well}^{-1}$  were seeded in six well plates. After 24 h, the medium was removed and the cells were washed once with phosphate-buffered saline and serum-deprived for 24 h at 37°C, 5%  $\text{CO}_2$ , 95% relative humidity in assay buffer (130 mM NaCl, 5.4 mM KCl, 1.8 mM  $\text{CaCl}_2$ , 0.8 mM  $\text{MgSO}_4$ , 0.9 mM  $\text{NaH}_2\text{PO}_4$ , 25 mM glucose, 20 mM HEPES, pH 7.4) containing 1% ( $\text{w v}^{-1}$ ) bovine serum albumin. At this stage, some wells were counted for initial cell number. The remaining wells were treated in triplicate with assay buffer alone (basal) or containing Fluo-4, AM (2  $\mu\text{M}$ ) and probenecid (0.1 mM). After 1 h at 37°C, 5%  $\text{CO}_2$ , 95% relative humidity, the plates were washed twice with assay buffer alone (basal) or with probenecid (0.1 mM) and finally resuspended in the same buffer (with 1  $\mu\text{M}$  SRIF-14 for nonbasal wells). After a further 5 h incubation, all wells were counted, this was carried out washing with phosphate-buffered saline and brief incubation with 0.5 ml trypsin (0.5 mg  $\text{ml}^{-1}$ )/EDTA (0.2 mg  $\text{ml}^{-1}$ ). Cells

were detached into culture medium and a 100  $\mu\text{l}$  aliquot was diluted 1:1 in Trypan blue stain before counting, using a cell counting chamber. The total number of cells per well was counted and expressed as a fraction of the number of cells counted in control wells at the start of the experiment.

### Analysis of data

Competition radioligand binding data were analysed by nonlinear regression curve fitting with the computer program SCTFIT (De Lean, 1979) and  $\text{pK}_d$  was derived from this fit. For FLIPR II data, the FLIPR II program directly performed background subtraction. Activation (% increase over basal) was then calculated for each well as  $100 \times ((\text{max peak obtained after agonist addition} - \text{basal}) / \text{basal})$ , where basal is the average of the first five values in each well obtained before agonist addition. For luciferase data, activation (% increase over basal) was calculated as  $100 \times ((\text{experimental} - \text{basal}) / \text{basal})$ . Concentration–response curves of luciferase expression data and intracellular  $\text{Ca}^{2+}$  data were analysed by nonlinear regression curve fitting using Graph Pad Prism<sup>TM</sup>. Values of maximal effect and  $\text{pEC}_{50}$  ( $-\log_{10}$  of the molar concentration producing half the maximal effect) were derived from this fit. Maximal responses of compounds were normalised to the maximal response induced by SRIF-14 alone (= 100%) and this was used as the  $E_{\text{max}}$  value (relative maximal effect). Results are given as means  $\pm$  s.e.m. Statistical analysis was also carried out using Graph Pad Prism and unless indicated, Student's *t*-test was used to analyse data.

### Drugs and materials

L 363,301 (c[Pro-Phe-DTrp-Lys-Thr-Phe]), SRIF-14 (Ala-Gly-c[Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys]-OH), SRIF-28 (Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys-Ala-Gly-c[Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Thr-Ser-Cys]-OH) and LTT-SRIF-28 ([Leu<sup>8</sup>,DTrp<sup>22</sup>,-Tyr<sup>25</sup>]SRIF-28; Ser-Ala-Asn-Ser-Asn-Pro-Ala-Leu-Ala-Pro-Arg-Glu-Arg-Lys-Ala-Gly-c[Cys-Lys-Asn-Phe-Phe-DTrp-Lys-Thr-Tyr-Thr-Ser-Cys]-OH) were from Bachem AG (Bubendorf, Switzerland). BIM 23027 (c[N-Me-Ala-Tyr-DTrp-Lys-Abu-Phe]), BIM 23052 (D-Phe-Phe-Phe-DTrp-Lys-Thr-Phe-Thr-NH<sub>2</sub>), cortistatin-14 (Pro-c[Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Ser-Ser-Cys]-Lys) and L 362,855 (c[Aha-Phe-Trp-DTrp-Lys-Thr-Phe]) were from Laboratoire Neosystem (Strasbourg, France). RC 160 (D-Phe-c[Cys-Tyr-DTrp-Lys-Val-Cys]-Trp-NH<sub>2</sub>) was from Peninsula Laboratories Inc. (San Carlos, CA, U.S.A.). L/DTrp<sup>8</sup>CYN 154806 (AcNH-4-NO<sub>2</sub>-Phe-c[DCys-Tyr-DTrp-Lys-Thr-Cys]-L/DTrp-NH<sub>2</sub>) and [<sup>125</sup>I][Tyr<sup>3</sup>]octreotide (D-Phe-c[Cys-(<sup>125</sup>I-Tyr)-DTrp-Lys-Thr-Cys]-Thr-OH) were custom synthesised by ANAWA AG (Wangen, Switzerland). BIM 23056 (D-Phe-Phe-Tyr-DTrp-Lys-Val-Phe-DNal-NH<sub>2</sub>), CGP 23996 (c[Asu-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Tyr-Thr-Ser]), octreotide (D-Phe-c[Cys-Phe-DTrp-Lys-Thr-Cys]-Thr-OH) and [Tyr<sup>3</sup>]octreotide (D-Phe-c[Cys-Tyr-DTrp-Lys-Thr-Cys]-Thr-OH) were synthesised at Novartis Pharma AG (Basel, Switzerland). Abbreviations: Abu = aminobutyric acid; Asu = amino suberic acid; Aha = 7-aminoheptanoic acid; Mpr = 3-mercaptopropionic acid; D-Nal = Naphthyl-D-Ala; Bzl = Benzylsubstituent. Nonpeptide compound **6** was synthesised at Novartis according to published procedures (Hay *et al.*, 2001a, b). DMEM/Ham's

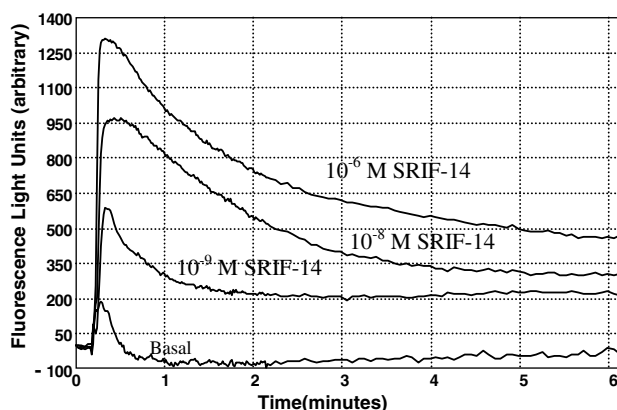
F12 nutrient mix, Foetal bovine serum, phosphate-buffered saline and trypsin/EDTA were purchased from Gibco BRL (Basel, Switzerland), G418 was purchased from Promega (Wallisellen, Switzerland), hygromycin B was purchased from Invitrogen AG (Basel, Switzerland), Fluo-4, AM was purchased from Molecular Probes (Leiden, The Netherlands) and 384-well black-walled culture plates were purchased from Becton Dickinson labware AG (Basel, Switzerland). Where not specified, chemicals were purchased from Sigma-Aldrich AG (Buchs, Switzerland).

## Results

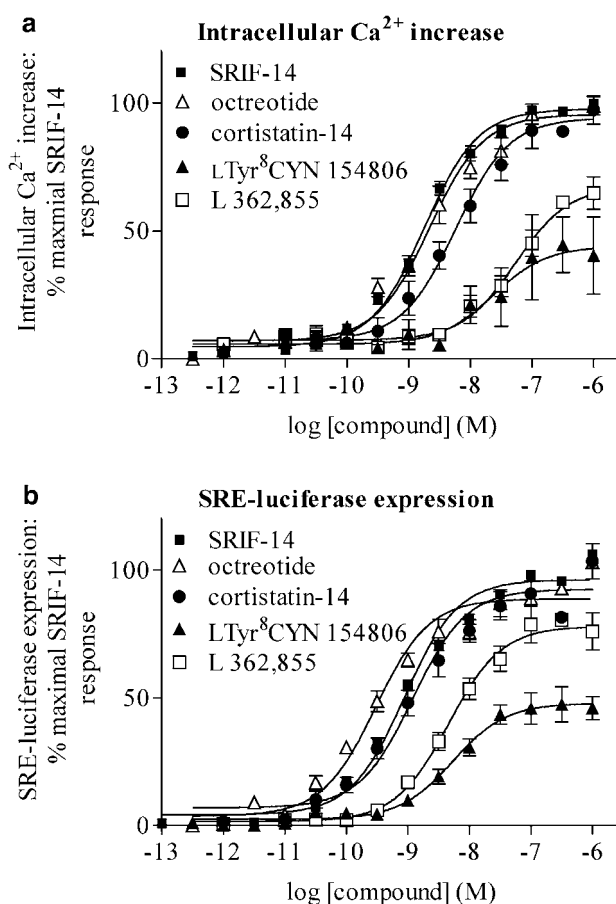
### Effects of SRIF and SRIF ligands on intracellular $Ca^{2+}$

SRIF-14 caused a rapid and transient increase in intracellular  $Ca^{2+}$  which peaked at 10–15 s and gradually declined over time, without reaching basal levels within the time frame of the experiment (6 min; Figure 1). SRIF-14 produced a maximal increase over basal (the first five fluorescence measurements before addition of SRIF-14) of  $169.7 \pm 5.6\%$  ( $n = 52$ ). The effect of SRIF-14 was concentration-dependent (Figure 2a), the calculated  $pEC_{50}$  was  $8.74 \pm 0.03$ , which is lower than the binding affinity at cell membranes ( $pK_d = 10.21 \pm 0.18$  determined with [ $^{125}I$ ][Tyr<sup>3</sup>]octreotide,  $n = 3$ ).

Cortistatin-14, SRIF-28 and synthetic analogues of SRIF were also tested for their ability to increase intracellular  $Ca^{2+}$  via the human  $ss_{t2}$  receptor (Figure 2a, Table 1). In general, compounds that are reported to be predominantly  $ss_{t2}$  selective (BIM 23027, L 363,301, compound 6 (Raynor *et al.*, 1993; Hay *et al.*, 2001a)), as well as those that are equally  $ss_{t2}$  and  $ss_{t5}$  selective ([Tyr<sup>3</sup>]octreotide, octreotide, RC 160; (Hannon *et al.*, 2002)) acted as full agonists (with the exception of the nonpeptide compound 6) and displayed high potencies that were similar to those of natural ligands and analogues (SRIF-14, SRIF-28, LTT-SRIF-28). CGP 23996 and cortistatin-14 had somewhat lower potency compared to the other analogues, whereas the predominantly  $ss_{t5}$  selective compounds (L 362,855, BIM 23052; (Hannon *et al.*, 2002)) as well as the two



**Figure 1** Increase in intracellular  $Ca^{2+}$  in CHO-K1 cells expressing human  $ss_{t2}$  receptors. Cells were incubated with Fluo-4 AM for 1 h. Following washing, plates were placed into a FLIPR™ where fluorescence intensity was determined before and after the addition of quadruplicates of the indicated concentrations of SRIF-14. Data show a representative trace, typical of 52 experiments.



**Figure 2** Effect of somatostatin agonists on intracellular  $Ca^{2+}$  modulation and SRE-luciferase reporter gene activity in CHO-K1 cells expressing human  $ss_{t2}$  receptors. Cells were incubated with Fluo-4 AM for 1 h. Following washing, plates were placed into a FLIPR™ where fluorescence intensity was determined before and after the addition of agonist (a). After a further 5 h incubation, cells were lysed and SRE-luciferase expression was determined (b). Data are expressed as % of maximal SRIF-14 response (= 100%) and represent the mean  $\pm$  s.e.m. of all experiments ( $n = 52$ ).

isoforms of the putative  $ss_{t2}$ -selective antagonist CYN 154806 were less potent and acted as partial agonists.

### Effects of SRIF and SRIF ligands on luciferase expression

Following the addition of agonist/antagonist and measurement of intracellular  $Ca^{2+}$  using FLIPR II, the microplates were incubated for 5 h. After this time, the cells were lysed and luciferase expression was determined. SRIF-14 concentration-dependently increased luciferase expression (Figure 2b) with a maximal increase over basal of  $378.5 \pm 18.3\%$  and a  $pEC_{50}$  of  $9.06 \pm 0.03$  ( $n = 52$ ).

As with intracellular  $Ca^{2+}$ , when a range of SRIF ligands were tested for their ability to increase luciferase expression, the  $ss_{t2}$ -selective, and  $ss_{t2}$  and  $ss_{t5}$  selective ligands displayed high potency which was similar to, or greater than, that of SRIF itself (Figure 2b, Table 1). The predominantly  $ss_{t5}$  selective ligands were about 10-fold less potent than SRIF and were partial agonists. The putative  $ss_{t2}$  selective antagonist

**Table 1** Comparison of agonist affinity, potency and efficacy in radioligand binding, intracellular Ca<sup>2+</sup> modulation and SRE-luciferase expression in CHO-K1 cells expressing human sst<sub>2</sub> receptors

| Compound                      | $[^{125}\text{I}][\text{Tyr}^3]$ octreotide binding<br>$\text{pK}_d$ | Intracellular Ca <sup>2+</sup> |                  | SRE-luciferase expression |                  |
|-------------------------------|--|--------------------------------|------------------|---------------------------|------------------|
|                               |  | $\text{pEC}_{50}$              | $E_{\text{max}}$ | $\text{pEC}_{50}$         | $E_{\text{max}}$ |
| SRIF-14                       | 10.21 ± 0.18   | 8.74 ± 0.03                    | 100              | 9.06 ± 0.03               | 100              |
| SRIF-28                       | 10.29 ± 0.24   | 8.74 ± 0.16                    | 85.4 ± 5.6       | 9.28 ± 0.09               | 91.0 ± 2.9       |
| LTT-SRIF-28                   | 10.25 ± 0.03   | 8.75 ± 0.16                    | 102.5 ± 5.7      | 9.16 ± 0.11               | 101.9 ± 3.5      |
| Cortistatin-14                | 9.04 ± 0.09  | 8.22 ± 0.10                    | 94.8 ± 3.4       | 8.92 ± 0.09               | 92.4 ± 2.7       |
| BIM 23027                     | 10.30 ± 0.07   | 8.96 ± 0.13                    | 86.7 ± 3.7       | 9.22 ± 0.07               | 97.7 ± 2.0       |
| L 363,301                     | 9.19 ± 0.08  | 8.60 ± 0.10                    | 89.8 ± 3.1       | 9.33 ± 0.11               | 81.3 ± 2.8       |
| Compound 6                    | 8.98 ± 0.14  | 8.58 ± 0.10                    | 76.1 ± 2.6       | 9.43 ± 0.09               | 83.8 ± 2.2       |
| Octreotide                    | 9.86 ± 0.21  | 8.66 ± 0.07                    | 95.5 ± 2.1       | 9.52 ± 0.06               | 88.9 ± 1.4       |
| [Tyr <sup>3</sup> ]octreotide | 9.91 ± 0.11  | 8.87 ± 0.12                    | 93.6 ± 3.9       | 9.75 ± 0.06               | 93.6 ± 1.98      |
| RC 160                        | 10.23 ± 0.33   | 8.60 ± 0.09                    | 98.6 ± 3.3       | 9.27 ± 0.08               | 101.3 ± 2.4      |
| CGP 23996                     | 9.62 ± 0.17  | 8.31 ± 0.12                    | 79.3 ± 3.7       | 8.59 ± 0.09               | 86.9 ± 2.9       |
| BIM 23052                     | 8.73 ± 0.03  | 7.13 ± 0.08                    | 75.6 ± 3.1       | 8.07 ± 0.05               | 82.6 ± 1.7       |
| L 362,855                     | 8.74 ± 0.04  | 7.22 ± 0.10                    | 70.0 ± 4.0       | 8.34 ± 0.08               | 78.1 ± 2.6       |
| dTyr <sup>8</sup> CYN 154806  | 8.48 ± 0.03  | 7.29 ± 0.32                    | 46.8 ± 6.8       | 8.36 ± 0.12               | 32.6 ± 1.4       |
| LTyr <sup>8</sup> CYN 154806  | 8.06 ± 0.06  | 7.44 ± 0.31                    | 48.8 ± 6.7       | 8.29 ± 0.10               | 47.3 ± 1.9       |
| BIM 23056                     | 6.65 ± 0.10  | CD                             | 23.5 ± 4.9       | CD                        | 27.0 ± 4.3       |

Potency is expressed as  $\text{pEC}_{50}$  (-log of the molar concentration required for a half maximal effect), efficacy as  $E_{\text{max}}$  (% of maximal SRIF-14 response) and binding affinity as  $\text{pK}_d$  (-log M) ± s.e.m. of 5–52 experiments. CD = cannot be determined.

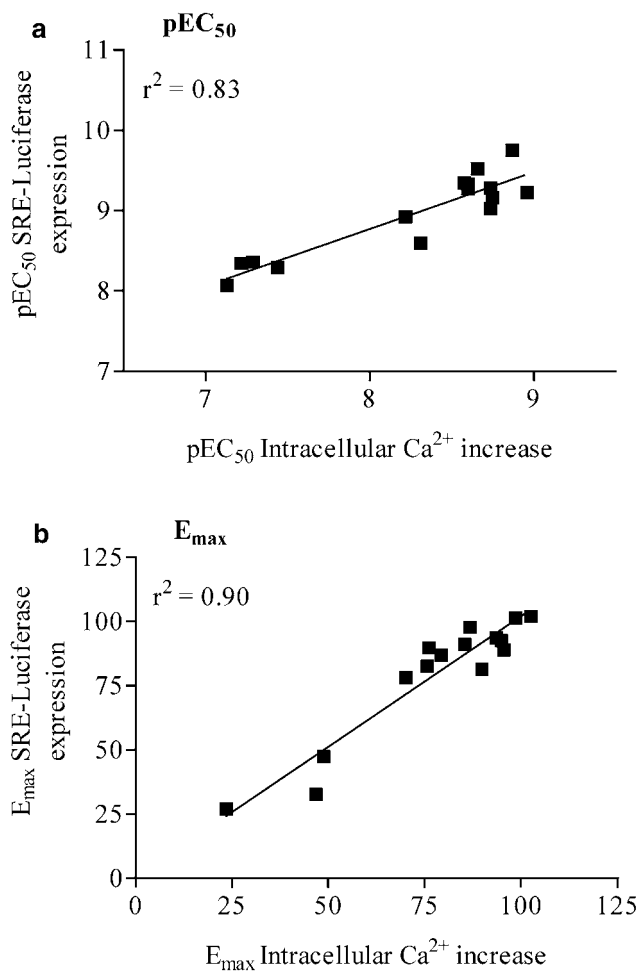
ligands dTyr<sup>8</sup>CYN 154806 and LTyr<sup>8</sup>CYN 154806, both acted as partial agonists.

#### Comparison of ligand effects in intracellular Ca<sup>2+</sup> and luciferase expression

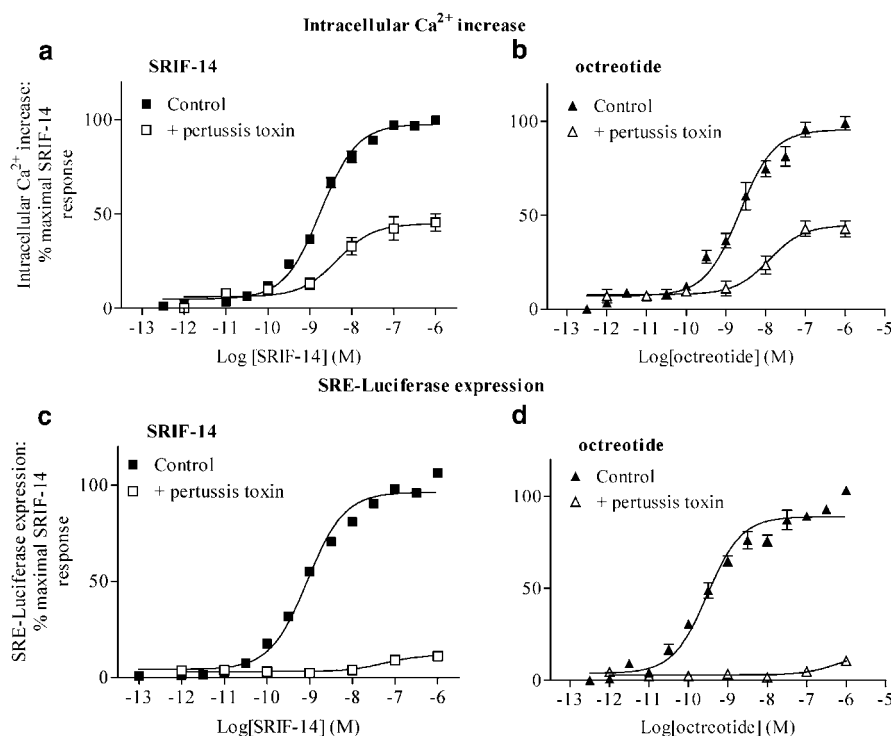
The pharmacological profiles (rank orders of potency and relative efficacy) determined in intracellular Ca<sup>2+</sup> and luciferase expression were compared. All compounds demonstrated significantly higher potency in stimulating luciferase expression than intracellular Ca<sup>2+</sup> ( $P = 0.003$ ) and this was as much as an eight fold difference for some compounds (e.g. [Tyr<sup>3</sup>]octreotide,  $\text{pEC}_{50} = 8.87 \pm 0.12$  and  $9.75 \pm 0.06$ ,  $n = 9$ , in intracellular Ca<sup>2+</sup> increase and luciferase expression, respectively). However, although rank orders of potency were different in the two assays, the overall correlation was good (Figure 3a). As with potency, rank orders of relative efficacy ( $E_{\text{max}}$ ) were different in the two assays, but the overall correlation and distribution of means was similar (Figure 3b). The potency values were also compared with binding affinity for [<sup>125</sup>I][Tyr<sup>3</sup>]octreotide labelled sst<sub>2</sub> receptors (Table 1). The binding affinity profile was rather less well correlated with potency profiles ( $r^2 = 0.72$  and  $0.49$  for intracellular Ca<sup>2+</sup> and luciferase expression, respectively).

#### Effects of pertussis toxin

Pertussis toxin pretreatment significantly reduced the maximal effect of both SRIF-14 and octreotide on intracellular Ca<sup>2+</sup> increase by 45–47% compared to the effect without pertussis toxin (Figure 4a and b,  $P = 0.046$  and  $0.037$ , respectively). The decrease in maximal effect was accompanied by a significantly decreased potency ( $\text{pEC}_{50}$  in the presence of pertussis toxin  $8.35 \pm 0.21$  and  $7.91 \pm 0.20$ ,  $n = 15$  and  $10$ , SRIF-14 and octreotide, respectively,  $P \leq 0.0025$ ). In contrast, pertussis toxin almost abolished the effects of SRIF-14 and octreotide on luciferase expression (Figure 4c and d;  $E_{\text{max}} = 4.8 \pm 0.4\%$  and  $1.3 \pm 0.4\%$  of maximal SRIF-14/octreotide response,



**Figure 3** Comparison of profiles of agonist potency ( $\text{pEC}_{50}$ , -log of the molar concentration required for a half-maximal effect) and relative efficacy ( $E_{\text{max}}$ , % of maximal SRIF-14 response) defined by intracellular Ca<sup>2+</sup> modulation and SRE-luciferase expression increase in the same microplate at human sst<sub>2</sub> receptors expressed in CHO-K1 cells. Data are from Table 1; correlation coefficients ( $r^2$ ) are indicated in both plots.



**Figure 4** Effect of pertussis toxin on SRIF-14/octreotide-stimulated intracellular  $\text{Ca}^{2+}$  modulation and SRE-luciferase reporter gene activity in CHO-K1 cells expressing human  $\text{sst}_2$  receptors. Cells that had been preincubated with or without pertussis toxin ( $100 \text{ ng ml}^{-1}$ , 18 h) were incubated with Fluo-4 AM for 1 h. Following washing, plates were placed into a FLIPR™ where fluorescence intensity was determined before and after the addition of the indicated concentrations of SRIF-14 (a) or octreotide (b). After a further 5 h, incubation, cells were lysed and SRE-luciferase expression was determined (c and d). Data are expressed as % of maximal SRIF-14 effect in the absence of pertussis toxin (= 100%) and represent the mean  $\pm$  s.e.m. of all experiments ( $n \geq 10$ ).

respectively, without pertussis toxin,  $n = 15$  and 10, respectively).

The effects of 10% (v/v) foetal calf serum on intracellular  $\text{Ca}^{2+}$  and luciferase expression were examined in the presence of pertussis toxin. This was a control to ensure that cells were functional in the presence of pertussis toxin and therefore that the effects of SRIF-14 and octreotide were reliably due to inhibition of the  $\text{G}_i/\text{G}_o$  protein and not simply due to decreased function of the cells. Pertussis toxin had no significant effect on either foetal calf serum-stimulated intracellular  $\text{Ca}^{2+}$  (% increase over basal:  $194.5 \pm 22.6$  and  $210.49 \pm 24.6$ ,  $n = 5$ , with and without pertussis toxin, respectively,  $P = 0.65$ ) or foetal calf serum-stimulated luciferase expression (% increase over basal:  $941.9 \pm 129.6$  and  $1349.7 \pm 210.0$ ,  $n = 5$ , with and without pertussis toxin, respectively,  $P = 0.14$ ).

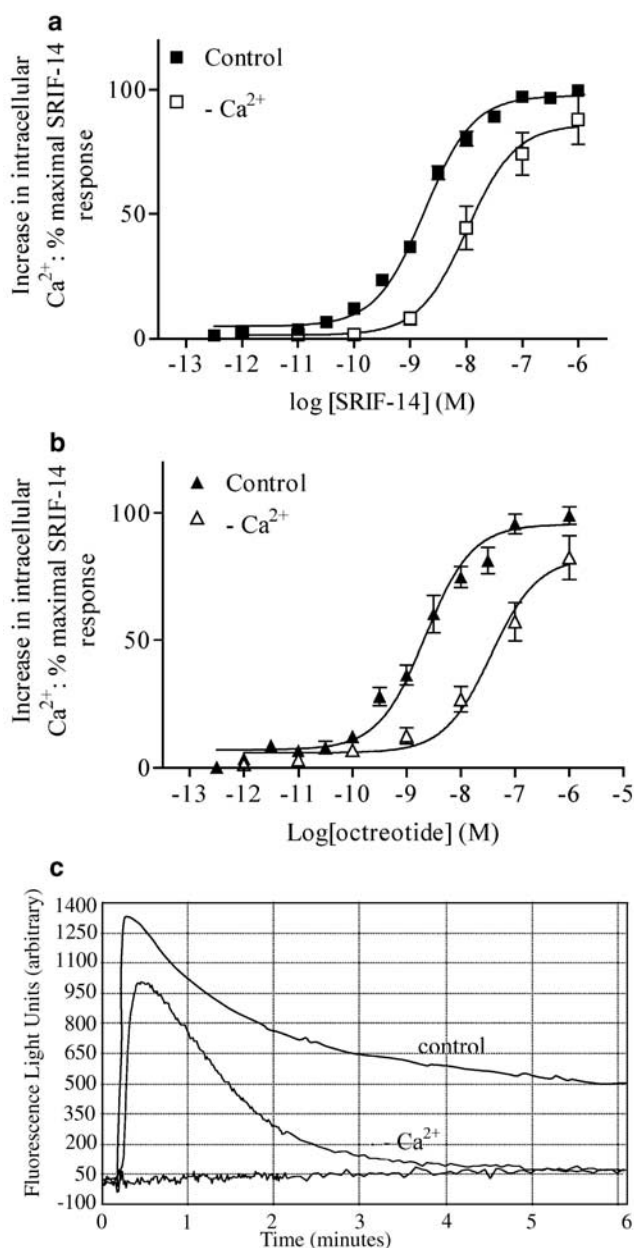
#### Mechanism of $\text{Ca}^{2+}$ increase

In order to determine the nature of the increase in intracellular  $\text{Ca}^{2+}$  via the human  $\text{sst}_2$  receptor, two sets of experiments were carried out. Firstly, the effects of SRIF-14 and octreotide were established in the absence of extracellular  $\text{Ca}^{2+}$ . Secondly, the effects of SRIF-14 and octreotide were established in the presence of the endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase inhibitor thapsigargin, which causes depletion of intracellular  $\text{Ca}^{2+}$  stores.

Removal of extracellular  $\text{Ca}^{2+}$  (and addition of 1 mM EGTA) caused a decrease in basal fluorescence ( $1606.0 \pm 38.6$

FLU,  $n = 5$ , without  $\text{Ca}^{2+}$  compared to  $4489.7 \pm 338.8$  FLU,  $n = 5$ , with  $\text{Ca}^{2+}$ ), but there was no difference in the maximum effect of SRIF-14 or octreotide to increase intracellular  $\text{Ca}^{2+}$  (Figure 5a and b;  $E_{\text{max}}$ :  $86.0 \pm 5.0$  and  $82.5 \pm 5.4\%$  of maximal SRIF-14 response in the presence of extracellular  $\text{Ca}^{2+}$ ,  $n = 14$  and 16, for SRIF-14 and octreotide, respectively;  $P \geq 0.38$ ). The potency of both SRIF-14 and octreotide to stimulate intracellular  $\text{Ca}^{2+}$  was reduced in the absence of extracellular  $\text{Ca}^{2+}$  ( $\text{pEC}_{50}$ :  $7.99 \pm 0.14$  and  $7.44 \pm 0.15$ ,  $n = 14$  and 16, SRIF-14 and octreotide, respectively,  $P < 0.0001$ ). The kinetics of the SRIF-14 response were changed with the fluorescence levels returning to basal within about 5 min. This was different to the kinetics in the presence of extracellular  $\text{Ca}^{2+}$  where a sustained plateau was observed (Figure 5c).

Removal of extracellular  $\text{Ca}^{2+}$  caused a decrease in the maximal effect of SRIF-14 and octreotide to stimulate luciferase expression ( $E_{\text{max}}$ :  $65.3 \pm 3.4$  and  $62.4 \pm 0.14\%$  of maximal SRIF-14 response in the presence of extracellular  $\text{Ca}^{2+}$ ,  $n = 14$  and 16, SRIF-14 and octreotide, respectively; data not shown). There was no difference in the potency of either compound in the absence of extracellular  $\text{Ca}^{2+}$  ( $\text{pEC}_{50}$ :  $9.08 \pm 0.17$  and  $9.65 \pm 0.14$ ,  $n = 14$  and 16, SRIF-14 and octreotide, respectively). However, it is likely that the decreased efficacy is due to a loss of function in the cells that were incubated for 5 h in the absence of extracellular  $\text{Ca}^{2+}$ . When tested for viability (by counting cell numbers), it was found that incubation in  $\text{Ca}^{2+}$ -free medium, either in basal conditions, or in the presence of SRIF-14, led to a significant decrease in cell number (fraction of initial cell number:



**Figure 5** Effect of removing extracellular  $\text{Ca}^{2+}$  on SRIF-14/octreotide-stimulated intracellular  $\text{Ca}^{2+}$  modulation in CHO-K1 cells expressing human  $\text{sst}_2$  receptors. Cells were incubated with Fluo-4 AM for 1 h. Plates were washed using buffer with or without  $\text{Ca}^{2+}$  and placed into a FLIPR™ where fluorescence intensity was determined before and after the addition of quadruplicates of SRIF-14/octreotide in the presence or absence of extracellular  $\text{Ca}^{2+}$ . EGTA (1 mM) was included in the  $\text{Ca}^{2+}$ -free buffer. Data in (a) and (b) are expressed as % of maximal SRIF-14 response in the presence of extracellular calcium (= 100%) and represent the mean  $\pm$  s.e.m. of all experiments ( $n \geq 16$ ). Data in (c) is a representative trace of kinetics of  $1 \mu\text{M}$  SRIF-14 in the presence and absence of extracellular calcium.

basal =  $0.74 \pm 0.03$ ,  $n = 22$ ; basal without extracellular  $\text{Ca}^{2+}$  =  $0.54 \pm 0.02$ ,  $n = 20$ ; SRIF-14 treated =  $0.85 \pm 0.03$ ,  $n = 24$ ; SRIF-14 treated without extracellular  $\text{Ca}^{2+}$  =  $0.59 \pm 0.03$ ,  $n = 24$ ; data not shown).

Thapsigargin ( $1 \mu\text{M}$ ) was preincubated with the cells for 1 h before determination of fluorescence in the FLIPR II. This was

carried out by addition to the loading buffer and all subsequent wash buffers. Thapsigargin preincubation had no effect on basal fluorescence levels ( $5317.7 \pm 719.9$  FLU,  $n = 3$ , without thapsigargin,  $6774.7 \pm 299.4$  FLU,  $n = 3$ , with thapsigargin,  $P = 0.135$ ); however, it abolished the effect of both SRIF-14 and octreotide on intracellular  $\text{Ca}^{2+}$  levels (Figure 6a and b). In contrast, when luciferase expression was measured 5 h later, while there was a drop in basal values ( $10.03 \pm 2.0$  RLU,  $n = 3$ , with thapsigargin compared to  $19.9 \pm 2.8$  RLU,  $n = 3$ , without thapsigargin), there was no difference ( $P \geq 0.46$ ) in maximum effect or potency of SRIF-14 and octreotide in the presence of thapsigargin (Figure 6c and d;  $E_{\text{max}}$ :  $107.9 \pm 3.6$  and  $88.3 \pm 2.7\%$  of maximal SRIF-14 response,  $\text{pEC}_{50}$ :  $8.92 \pm 0.10$  and  $9.50 \pm 0.09$ ,  $n = 6$  and 4, SRIF-14 and octreotide, respectively).

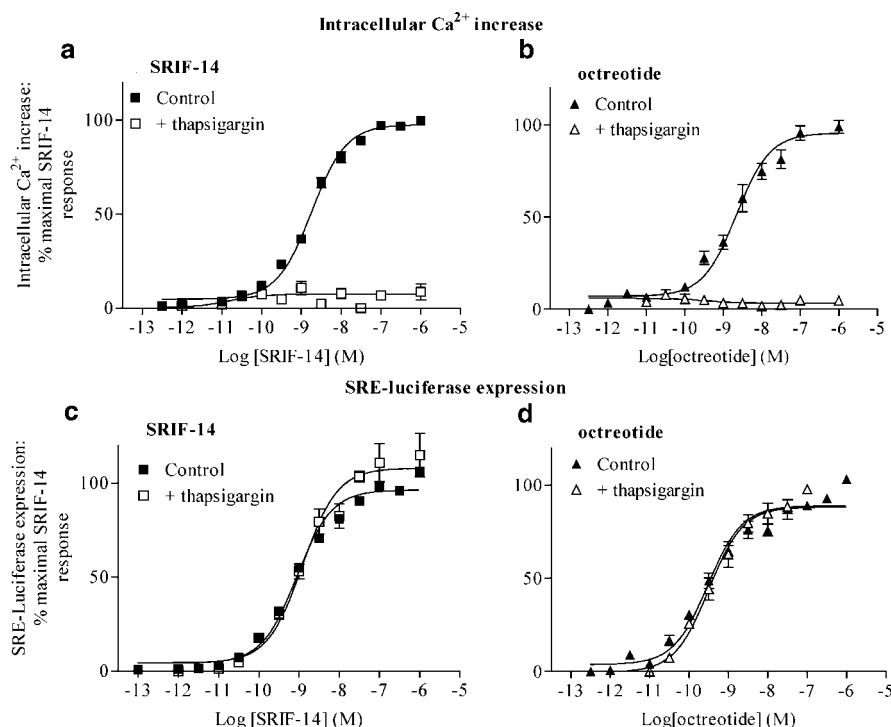
## Discussion

Functional effects of SRIF receptors have been extensively studied *in vitro* in both natural and recombinant systems *via* determination of diverse transduction parameters including adenylyl cyclase inhibition, phospholipase C stimulation, elevation of intracellular  $\text{Ca}^{2+}$  concentrations, extracellular acidification rates using microphysiometry,  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  binding, MAP kinase activation and tyrosine phosphatase activity to name but a few (Florio & Schettini, 1996; Patel, 1999). However, discrepancies often occur between the effects of SRIF ligands at a given receptor subtype, depending on the cell line, binding test, or second messenger studied. In addition, agonists can have rather different effects at a particular receptor subtype depending on the functional assay, suggesting the existence of multiple agonist-specific receptor conformations (Siehler *et al.*, 1999; Siehler & Hoyer, 1999a, b). It has been suggested that signal transduction pathways activated by the same SRIF receptor may apparently display a different profile (Siehler *et al.*, 1999; Siehler & Hoyer, 1999a–c; Nunn *et al.*, 2002), although it may be argued that experimental conditions are usually different when comparing binding studies and the various transduction assays.

The aim of this study was to examine two functional responses after a single ligand-receptor binding event. The development of an assay in which two responses can be determined in the same microplate confers a number of advantages over measurement of a single functional parameter. Firstly, when considering pharmacology, it removes the possibility of differences being observed due to different assay conditions (pH, temperature, cell number, etc.). Secondly, from a drug discovery perspective, simultaneous measurement of pharmacological responses reduces reagent cost and workload and offers the possibility to identify hits acting in different pathways following receptor activation.

In this study, we have examined SRIF-ligand-induced increases in (i) intracellular  $\text{Ca}^{2+}$  using FLIPR II, and (ii) SRE-driven luciferase expression at human  $\text{sst}_2$  receptors stably expressed in CHO-K1 cells. We show that SRIF concentration-dependently increases both parameters with comparable potencies in the two assays.

Determination of luciferase expression was possible as a marker of functional activation of the  $\text{sst}_2$  receptor, since the CHO-K1 cells used to express the receptor were also



**Figure 6** Effect of thapsigargin on SRIF-14/octreotide-stimulated intracellular  $\text{Ca}^{2+}$  modulation and SRE-luciferase reporter gene activity in CHO-K1 cells expressing human  $\text{sst}_2$  receptors. Cells were incubated with Fluo-4 AM, in the presence or absence of thapsigargin (1  $\mu\text{M}$ ) for 1 h. Following washing, plates were placed into a FLIPR™ where fluorescence intensity was determined before and after the addition of the indicated concentrations of SRIF-14 (a) or octreotide (b). After a further 5 h incubation, cells were lysed and SRE-luciferase expression was determined (c and d). Data are expressed as % of maximal SRIF-14 effect (= 100%) and represent the mean  $\pm$  s.e.m. of all experiments ( $n \geq 4$ ).

transfected with the luciferase reporter gene under the control of the SRE promoter sequence. This sequence, which is associated with a variety of genes, is regulated by transcription factors that are constitutively bound to the DNA. These transcription factors are rapidly phosphorylated and activated in response to many extracellular signals, including ligands that act at G protein-coupled receptors (Hill & Treisman, 1995; Treisman, 1995; Chai & Tarnawski, 2002). In particular, transcriptional activation of the SRE requires the binding of two transcription factors – the serum response factor (SRF) and the ternary complex factor (TCF; Treisman, 1994; Price *et al.*, 1995). TCF phosphorylation *via* MAP kinases is necessary for it to bind SRF, and depending on the cell type and growth factor involved, all three families of MAP kinases (extracellular signal-regulated kinases, stress-activated protein kinases and p38) have been shown to be able to activate the TCF *via* different pathways (Treisman, 1994; 1995; Gille *et al.*, 1995; Price *et al.*, 1995; Whitmarsh *et al.*, 1995; 1997). In the present study and in previous studies in our laboratory, we have shown that determination of SRIF-induced luciferase expression produces a sensitive marker of agonist efficacy that differentiates between full and partial agonists (Feuerbach *et al.*, 2000; Nunn *et al.*, 2002; 2003a–c).

SRIF has previously been shown to be able to increase intracellular  $\text{Ca}^{2+}$  *via*  $\text{sst}_2$  receptors in recombinant cell lines including COS-7 (Akbar *et al.*, 1994; Tomura *et al.*,

1994),  $\text{F}_4\text{C}_1$  pituitary cells (Chen *et al.*, 1997), HEK-293 (Chen & Tashjian, 1994), and to a very small extent in CCL39 Chinese hamster lung fibroblasts (Siehler & Hoyer, 1999c). The effect has also been demonstrated in native systems including cultured mouse striatal astrocytes (Marin *et al.*, 1991), rat AR42J pancreas cells (Taylor, 1995), salamander retinal cells (Akopian *et al.*, 2000), aortic smooth muscle (Romoser *et al.*, 2001) and B lymphoblasts, where similar kinetics to those of this study were observed (Roskopf *et al.*, 2003). In the majority of studies, the increase in intracellular  $\text{Ca}^{2+}$  by SRIF parallels an increase in intracellular inositol phosphates and therefore is likely to be mediated *via* phospholipase C stimulation (Akbar *et al.*, 1994; Tomura *et al.*, 1994; Chen & Tashjian, 1994; Chen *et al.*, 1997; Siehler & Hoyer, 1999c; Romoser *et al.*, 2001; Roskopf *et al.*, 2003). Although, in cultured striatal astrocytes and rat AR42J pancreas cells, SRIF-induced rises in intracellular  $\text{Ca}^{2+}$  are independent of increased intracellular inositol phosphates (Marin *et al.*, 1991; Taylor, 1995).

Good correlation of potencies and efficacies of the natural and synthetic SRIF ligands tested in the two assays suggests little difference in agonist effects in either response. All compounds stimulated luciferase expression with higher potency than intracellular  $\text{Ca}^{2+}$ , suggesting that stimulation of luciferase expression may be affected more by receptor reserve, as was shown previously when comparing luciferase expression and [ $^{35}\text{S}$ ]GTP $\gamma$ S binding



in CCL39 cells expressing human sst<sub>2</sub> receptors (Siehler & Hoyer, 1999a; Nunn *et al.*, 2003a, c). From these data, it seems that the agonist effect in the two assays is certainly not superimposable, but is highly correlated and suggests that receptor activation by each ligand is producing a similar effect on both stimulation of intracellular Ca<sup>2+</sup> and luciferase expression.

Functional potency (pEC<sub>50</sub>) in the two assays was lower than pK<sub>d</sub> determined in binding. The reason for this may be due to differences in assay conditions (whole cells vs membranes, different Na<sup>+</sup> concentrations, etc.). However, it may also be due to a low coupling efficiency of the receptors. We have previously shown that agonist radioligand binding at the sst<sub>2</sub> receptor is only partially sensitive to the nonhydrolysable GTP analogue GppNHp (5'-guanylyl-imidodiphosphate; Siehler *et al.*, 1999), which suggests that binding affinities may represent receptors that are both coupled and uncoupled to G proteins, whereas functional assays will represent only ligand binding to functional receptors.

In order to determine the G proteins involved in the two responses, the effect of pertussis toxin was examined. This showed that luciferase expression is mediated solely *via* activation of G<sub>i</sub>/G<sub>o</sub> proteins (as we have shown previously, Nunn *et al.*, 2002). In contrast, intracellular Ca<sup>2+</sup> stimulation appears to be only partially mediated by G<sub>i</sub>/G<sub>o</sub> proteins. This is in agreement with previous findings that stimulation of phospholipase C, leading to an increase in intracellular Ca<sup>2+</sup> by SRIF receptors, is only partially inhibited by pertussis toxin in COS-7 cells expressing human sst<sub>2</sub>, sst<sub>3</sub> and sst<sub>5</sub> receptors (Akbar *et al.*, 1994; Tomura *et al.*, 1994), F<sub>4</sub>C<sub>1</sub> cells expressing sst<sub>2</sub> receptors (Chen *et al.*, 1997), CCL39 cells expressing sst<sub>2</sub>, sst<sub>3</sub> and sst<sub>5</sub> (Siehler & Hoyer, 1999c) and in B lymphoblasts which predominantly express sst<sub>2A</sub> receptors (Roskopf *et al.*, 2003). The pertussis toxin-insensitive part of the effect has been suggested to be due to stimulation of G<sub>q</sub> (or members of the G<sub>q</sub>/G<sub>11</sub> family) by SRIF (Akbar *et al.*, 1994; Tomura *et al.*, 1994; Chen *et al.*, 1997; Siehler & Hoyer, 1999c; Roskopf *et al.*, 2003), although this has never been explicitly proven. The potency and efficacy of SRIF-14 and octreotide to stimulate intracellular Ca<sup>2+</sup> were reduced in the presence of pertussis toxin, which may show that coupling to G<sub>i</sub>/G<sub>o</sub> proteins occurs at lower ligand concentrations than coupling to other G proteins as shown in COS-7 cells expressing recombinant SRIF receptors (Akbar *et al.*, 1994).

Since SRIF has previously been shown to be able to increase intracellular Ca<sup>2+</sup> in nonexcitable cells *via* both influx of extracellular Ca<sup>2+</sup> and release from intracellular stores (Marin *et al.*, 1991; Akbar *et al.*, 1994; Chen & Tashjian, 1994; Tomura *et al.*, 1994; Taylor, 1995; Chen *et al.*, 1997; Siehler & Hoyer, 1999c; Romoser *et al.*, 2001; Roskopf *et al.*, 2003), the mechanism by which SRIF, *via* sst<sub>2</sub>, produces an increase in intracellular Ca<sup>2+</sup> in these cells was examined. As luciferase expression was also measured in each assay, it was interesting to compare the effects of these manipulations on luciferase expression.

The complete block of SRIF-induced Ca<sup>2+</sup> increase with thapsigargin suggests that release of Ca<sup>2+</sup> from intracellular stores is the primary mechanism involved. However, removal of extracellular Ca<sup>2+</sup> also produced a small decrease in the response and this was accompanied by decreased potency and altered kinetics. The reason

for decreased SRIF potency is currently unclear. It is unlikely to be due to a direct effect of calcium on the receptor conformation, since the potency of these two peptides in luciferase expression experiments remain unchanged. One plausible explanation could be that the kinetics of SRIF binding to its receptor are highly dependent on external calcium under nonequilibrium conditions of FLIPR experiments in contrast to downstream luciferase events. Altogether, these data suggest that both intracellular and extracellular Ca<sup>2+</sup> are involved in the regulation of intracellular Ca<sup>2+</sup> by SRIF, with the initial peak being mainly due to mobilisation of intracellular Ca<sup>2+</sup> stores and the sustained plateau being the result of Ca<sup>2+</sup> influx across the plasma membrane. This is in accordance with the accepted model of agonist-stimulated intracellular Ca<sup>2+</sup> in nonexcitable cells (Berridge, 1993; Wilkinson *et al.*, 1997; Smart *et al.*, 1999); since both the initial peak and the sustained plateau are abolished by thapsigargin, it is likely that the Ca<sup>2+</sup> influx is secondary to the mobilisation of intracellular Ca<sup>2+</sup> and therefore is probably due to a capacitative entry mechanism (Berridge, 1997; Taylor & Broad, 1998). It has been shown that sst<sub>2</sub> expressed in COS-7 cells, sst<sub>5</sub> expressed in CHO-K1 cells and endogenous sst<sub>2A</sub> in B lymphoblasts increase intracellular Ca<sup>2+</sup> by mechanisms involving both intracellular and extracellular Ca<sup>2+</sup> as reported here (Tomura *et al.*, 1994; Wilkinson *et al.*, 1997; Roskopf *et al.*, 2003). In contrast, in rat pancreas AR42J cells, SRIF-induced increase in intracellular Ca<sup>2+</sup>, mediated by sst<sub>2</sub>, occurs solely *via* influx from extracellular Ca<sup>2+</sup> (Taylor, 1995).

The luciferase response is apparently independent of intracellular Ca<sup>2+</sup> changes since thapsigargin had no effect. This is not surprising since the mechanisms underlying SRE-mediated events are distinct from those taking place during calcium mobilisation. SRE activation is likely to involve stimulation of the MAP kinase pathway and is probably much less dependent upon intracellular calcium. However, it was not possible to establish the effect of removal of extracellular Ca<sup>2+</sup> on luciferase expression since the cells did not function properly without extracellular Ca<sup>2+</sup> for the long incubation time required for luciferase expression determination.

In conclusion, this study describes the determination of two functional responses simultaneously at the human sst<sub>2</sub> receptor expressed in CHO-K1 cells. It shows that ligand binding to the sst<sub>2</sub> receptor can induce increases in both intracellular Ca<sup>2+</sup> and luciferase expression. Comparison of the profiles of the two responses suggest that the sst<sub>2</sub> receptor is acting classically with parallel efficacy and potency profiles for luciferase expression and intracellular Ca<sup>2+</sup>, although luciferase expression may show higher receptor reserve or better coupling, having higher potency than intracellular Ca<sup>2+</sup> stimulation. The data show that when determined simultaneously, the effects of SRIF ligands on intracellular Ca<sup>2+</sup> and luciferase expression display a similar recognition profile, suggesting that the ligand/receptor/G protein/effector interaction is similar for the two parameters despite possible differences in the G proteins and pathways involved.

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