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Comparison of functional profiles at human recombinant somatostatin sst_2 receptor: simultaneous determination of intracellular Ca^{2+} 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_1–sst_5) that modulate multiple cellular effectors. The aim of this study was to compare two functional effects of the human sst_2 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_{50} 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 ($pEC_{50} = 9.06 \pm 0.03$, $n = 52$).

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 ($r^2 = 0.83$ and 0.90, pEC_{50} and E_{max}, 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_2 receptors in CHO-K1 cells. The increase in luciferase is mediated *via* G_i/G_o while intracellular calcium increase is mediated by both G_i/G_o 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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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_1–sst_5; Hoyer *et al*., 1995). According to structural and pharmacological similarities, the five receptors can be classified in two groups – $SRIF_1$ which consists of sst_2, sst_3 and sst_5, and $SRIF_2$ which consists of sst_1 and sst_4 (Hoyer *et al*., 1995; Hannon *et al*., 2002).

All five receptors are coupled to adenylyl cyclase *via* G_i/G_o 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^{2+} channels, K^+ 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, [^{35}S]GTPγS 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 sst_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; Rosskopf *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 sst_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 sst_2 receptors at levels which can be considered to be close to physiological (120 fmol mg^{-1} protein compared to 105 fmol mg^{-1} protein in rat cortex; Schoeffter *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^{-1}) foetal bovine serum at 37°C, 5% CO_2, 95% relative humidity. Low concentrations of selection antibiotics were also included in the culture medium (geneticin sulphate (G418), 100 μg ml^{-1} and hygromycin B, 100 μg ml^{-1}). The cells were passaged every 2 days by washing with phosphate-buffered saline and brief incubation with trypsin (0.5 mg ml^{-1})/EDTA (0.2 mg ml^{-1}). For storage, the cells were resuspended in medium containing 10% (v v^{-1}) dimethyl sulphoxide and 20% (v v^{-1}) 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°C for 5 min at 2500 × *g*. The cell pellet was either stored at −80°C or used directly. Before the experiments, cell membranes were resuspended in binding assay buffer (10 mM HEPES, 0.5% (w v^{-1}) bovine serum albumin, pH 7.5) by homogenisation with a Polytron homogeniser at 50 Hz for 20 s.

Cell homogenate (150 μl) was incubated with 50 μl of radioligand (2175 Ci mmol^{-1}, 25–35 pM final concentration), in binding assay buffer containing 5 mM $MgCl_2$, and the protease inhibitor bacitracin (5 μg ml^{-1}), and either 50 μl assay buffer (total binding); SRIF-14 (10 μM; 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^{-1}) 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 β scintillation counter (Packard TopCount) using scintillation liquid (80% counting efficiency).

Codetermination of intracellular Ca^{2+} and luciferase expression

Cells were seeded at 9000 cells 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 $CaCl_2$, 0.8 mM $MgSO_4$, 0.9 mM NaH_2PO_4, 25 mM glucose, 20 mM HEPES, pH 7.4) containing bovine serum albumin (1% w v^{-1}). Where pertussis toxin was used it was included in this buffer (100 ng ml^{-1}).

On the day of the experiment, the cells were treated with assay buffer containing the Ca^{2+}-sensitive fluorescent dye Fluo4-AM (2 μM), and probenecid (0.1 mM). Where thapsigargin was used, it was included in this buffer (1 μM). 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 Ca^{2+}, buffer without 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% CO_2, 95% relative humidity), the buffer containing test compounds was removed and cells were lysed in 10 μl lysis buffer (25 mM Tris-phosphate, 2 mM DL-dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% (v v^{-1}) glycerol, 1% (v v^{-1}) triton-X, pH 7.8). A volume of 20 μl luciferase assay buffer (20 mM Tris, 1.07 mM $Mg(CO_3)_4$, 2.67 mM $MgSO_4$, 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™ ultraHTS microplate imager (Perkin Elmer Life Sciences; exposure time 30 s).

Cell growth assay

A total of 500,000 cells 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% CO_2, 95% relative humidity in assay buffer (130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.9 mM NaH_2PO_4, 25 mM glucose, 20 mM HEPES, pH 7.4) containing 1% (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 μM) and probenecid (0.1 mM). After 1 h at 37°C, 5% 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 μM 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 ml^{-1})/EDTA (0.2 mg ml^{-1}). Cells were detached into culture medium and a 100 μl 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 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 × ((max peak obtained after agonist addition−basal)/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 × ((experimental-basal)/basal). Concentration–response curves of luciferase expression data and intracellular Ca^{2+} data were analysed by nonlinear regression curve fitting using Graph Pad Prism™. Values of maximal effect and 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_{max} value (relative maximal effect). Results are given as means ± 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-Phe-Thr-Ser-Cys]-OH) and LTT-SRIF-28 ([Leu^8,DTrp^{22},-Tyr^{25}]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 (DPhe-Phe-Phe-DTrp-Lys-Thr-Phe-Thr-NH_2), 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 (DPhe-c[Cys-Tyr-DTrp-Lys-Val-Cys]-Trp-NH_2) was from Peninsula Laboratories Inc. (San Carlos, CA, U.S.A.). L/DTyr^{8}CYN 154806 (AcNH-4-NO_2-Phe-c[DCys-Tyr-DTrp-Lys-Thr-Cys]-L/DTyr-NH_2) and [^{125}I][Tyr^3]octreotide (DPhe-c[Cys-(^{125}I-Tyr)-DTrp-Lys-Thr-Cys]-Thr-OH) were custom synthesised by ANAWA AG (Wangen, Switzerland). BIM 23056 (DPhe-Phe-Tyr-DTrp-Lys-Val-Phe-DNal-NH_2), CGP 23996 (c[Asu-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Tyr-Thr-Ser]), octreotide (DPhe-c[Cys-Phe-DTrp-Lys-Thr-Cys]-Thr-OH) and [Tyr^3]octreotide (DPhe-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 ± 0.03, which is lower than the binding affinity at cell membranes ($pK_d = 10.21 \pm 0.18$ determined with [^{125}I][Tyr^3]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 sst_2 receptor (Figure 2a, Table 1). In general, compounds that are reported to be predominantly sst_2 selective (BIM 23027, L 363,301, compound **6** (Raynor *et al*., 1993; Hay *et al*., 2001a)), as well as those that are equally sst_2 and sst_5 selective ([Tyr^3]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 sst_5 selective compounds (L 362,855, BIM 23052; (Hannon *et al*., 2002)) as well as the two isoforms of the putative sst_2-selective antagonist CYN 154806 were less potent and acted as partial agonists.

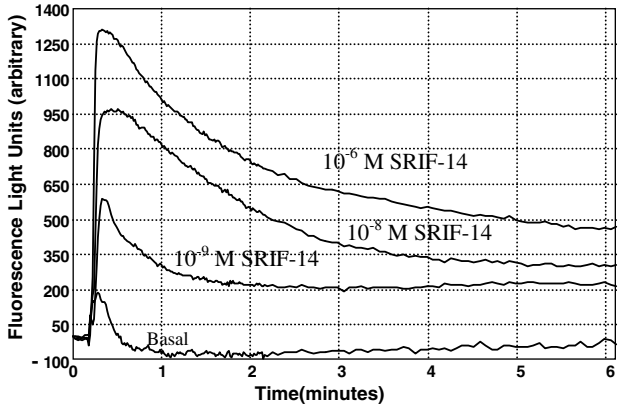

Figure 1 Increase in intracellular Ca^{2+} in CHO-K1 cells expressing human sst_2 receptors. Cells were incubated with Fluo-4 AM for 1 h. Following washing, plates were placed into a FLIPR™ where fluorescence intensity was determined before and after the addition of quadruplicates of the indicated concentrations of SRIF-14. Data show a representative trace, typical of 52 experiments.

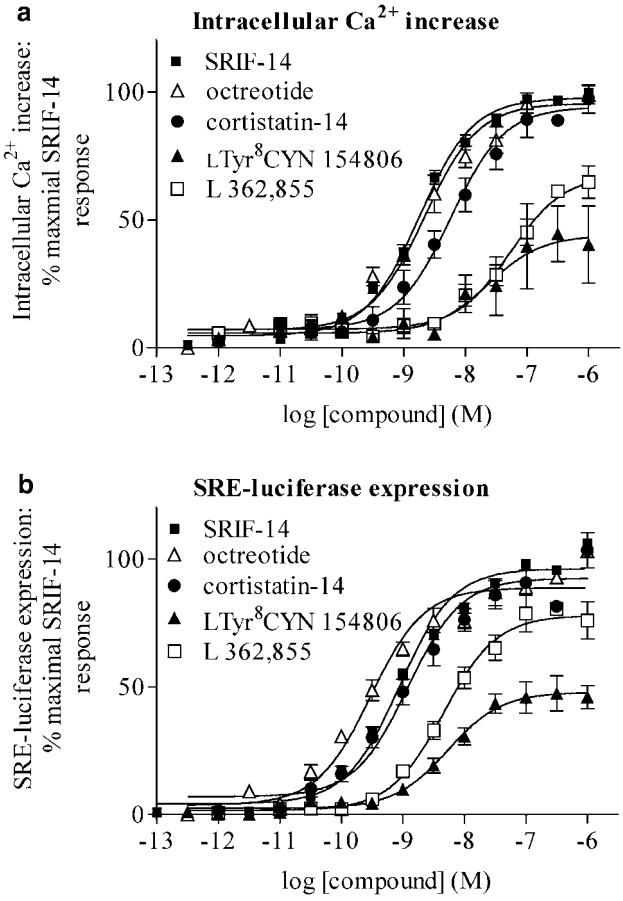

Figure 2 Effect of somatostatin agonists on intracellular Ca^{2+} modulation and SRE-luciferase reporter gene activity in CHO-K1 cells expressing human sst_2 receptors. Cells were incubated with Fluo-4 AM for 1 h. Following washing, plates were placed into a FLIPR™ where fluorescence intensity was determined 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 ± s.e.m. of all experiments ($n = 52$).

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 ± 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 sst_2-selective, and sst_2 and sst_5 selective ligands displayed high potency which was similar to, or greater than, that of SRIF itself (Figure 2b, Table 1). The predominantly sst_5 selective ligands were about 10-fold less potent than SRIF and were partial agonists. The putative sst_2 selective antagonist

Table 1 Comparison of agonist affinity, potency and efficacy in radioligand binding, intracellular Ca^{2+} modulation and SRE-luciferase expression in CHO-K1 cells expressing human sst_2 receptors

	[^{125}I][Tyr^3] octreotide binding	*Intracellular Ca^{2+}*		*SRE-luciferase expression*	
Compound	*pK_d*	*pEC_{50}*	*E_{max}*	*pEC_{50}*	*E_{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^3]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^{8}CYN 154806	8.48±0.03	7.29±0.32	46.8±6.8	8.36±0.12	32.6±1.4
lTyr^{8}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 pEC_{50} (-log of the molar concentration required for a half maximal effect), efficacy as E_{max} (% of maximal SRIF-14 response) and binding affinity as pK_d (-log M)±s.e.m. of 5–52 experiments. CD = cannot be determined.

ligands dTyr^{8}CYN 154806 and lTyr8CYN 154806, both acted as partial agonists.

Comparison of ligand effects in intracellular Ca^{2+} and luciferase expression

The pharmacological profiles (rank orders of potency and relative efficacy) determined in intracellular Ca^{2+} and luciferase expression were compared. All compounds demonstrated significantly higher potency in stimulating luciferase expression than intracellular Ca^{2+} ($P=0.003$) and this was as much as an eight fold difference for some compounds (e.g. [Tyr^3]octreotide, $pEC_{50}=8.87\pm0.12$ and 9.75 ± 0.06, $n=9$, in intracellular Ca^{2+} 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_{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 [^{125}I][Tyr^3]octreotide labelled sst_2 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^{2+} 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^{2+} 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 (pEC_{50} in the presence of pertussis toxin 8.35 ± 0.21 and 7.91 ± 0.20, $n=15$ and 10, SRIF-14 and octreotide, respectively, $P\leqslant0.0025$). In contrast, pertussis toxin almost abolished the effects of SRIF-14 and octreotide on luciferase expression (Figure 4c and d; $E_{max}=4.8\pm0.4\%$ and $1.3\pm0.4\%$ of maximal SRIF-14/octreotide response,

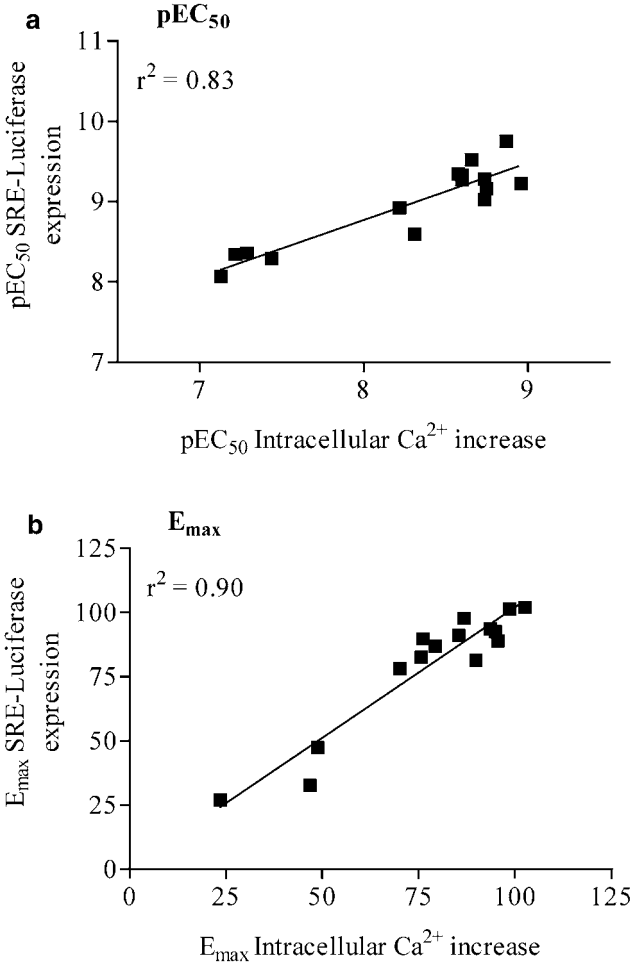

Figure 3 Comparison of profiles of agonist potency (pEC_{50}, -log of the molar concentration required for a half-maximal effect) and relative efficacy (E_{max}, % of maximal SRIF-14 response) defined by intracellular Ca^{2+} modulation and SRE-luciferase expression increase in the same microplate at human sst_2 receptors expressed in CHO-K1 cells. Data are from Table 1; correlation coefficients (r^2) are indicated in both plots.

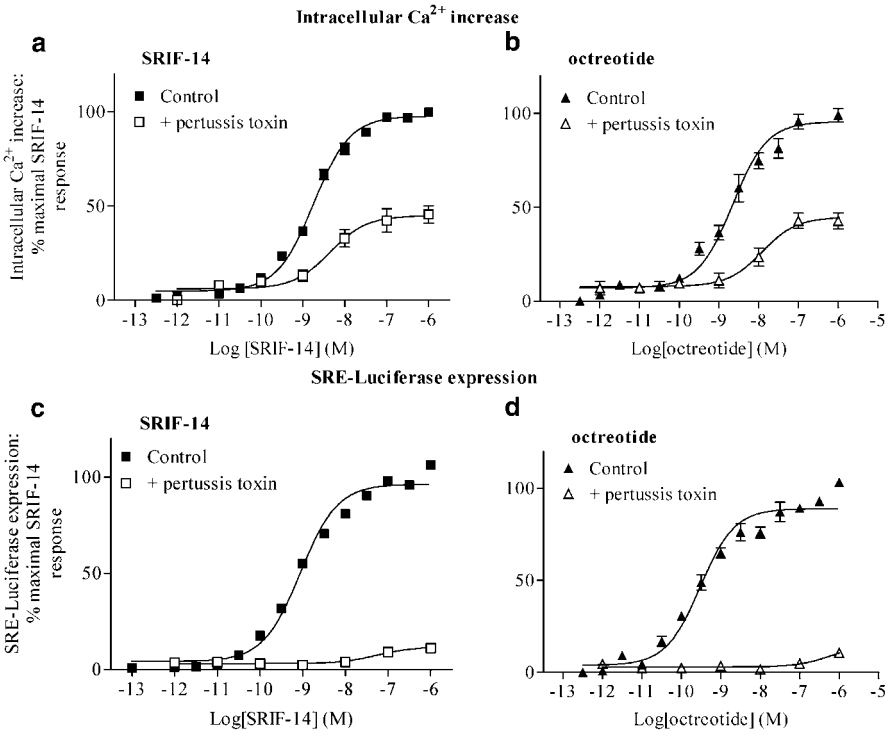

Figure 4 Effect of pertussis toxin on SRIF-14/octreotide-stimulated intracellular Ca^{2+} modulation and SRE-luciferase reporter gene activity in CHO-K1 cells expressing human sst_2 receptors. Cells that had been preincubated with or without pertussis toxin ($100\,ng\,ml^{-1}$, 18 h) were incubated with 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±s.e.m. of all experiments ($n \geqslant 10$).

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

The effects of 10% ($v\,v^{-1}$) foetal calf serum on intracellular 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 G_i/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 Ca^{2+} (% increase over basal: 194.5±22.6 and 210.49±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±129.6 and 1349.7±210.0, $n=5$, with and without pertussis toxin, respectively, $P=0.14$).

Mechanism of Ca^{2+} increase

In order to determine the nature of the increase in intracellular Ca^{2+} *via* the human 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 Ca^{2+}. Secondly, the effects of SRIF-14 and octreotide were established in the presence of the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin, which causes depletion of intracellular Ca^{2+} stores.

Removal of extracellular Ca^{2+} (and addition of 1 mM EGTA) caused a decrease in basal fluorescence (1606.0±38.6 FLU, $n=5$, without Ca^{2+} compared to 4489.7±338.8 FLU, $n=5$, with Ca^{2+}), but there was no difference in the maximum effect of SRIF-14 or octreotide to increase intracellular Ca^{2+} (Figure 5a and b; E_{max}: 86.0±5.0 and 82.5±5.4% of maximal SRIF-14 response in the presence of extracellular Ca^{2+}, $n=14$ and 16, for SRIF-14 and octreotide, respectively; $P \geqslant 0.38$). The potency of both SRIF-14 and octreotide to stimulate intracellular Ca^{2+} was reduced in the absence of extracellular Ca^{2+} (pEC_{50}: 7.99±0.14 and 7.44±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 Ca^{2+} where a sustained plateau was observed (Figure 5c).

Removal of extracellular Ca^{2+} caused a decrease in the maximal effect of SRIF-14 and octreotide to stimulate luciferase expression (E_{max}: 65.3±3.4 and 62.4±0.14% of maximal SRIF-14 response in the presence of extracellular 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 Ca^{2+} (pEC_{50}: 9.08±0.17 and 9.65±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 Ca^{2+}. When tested for viability (by counting cell numbers), it was found that incubation in 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:

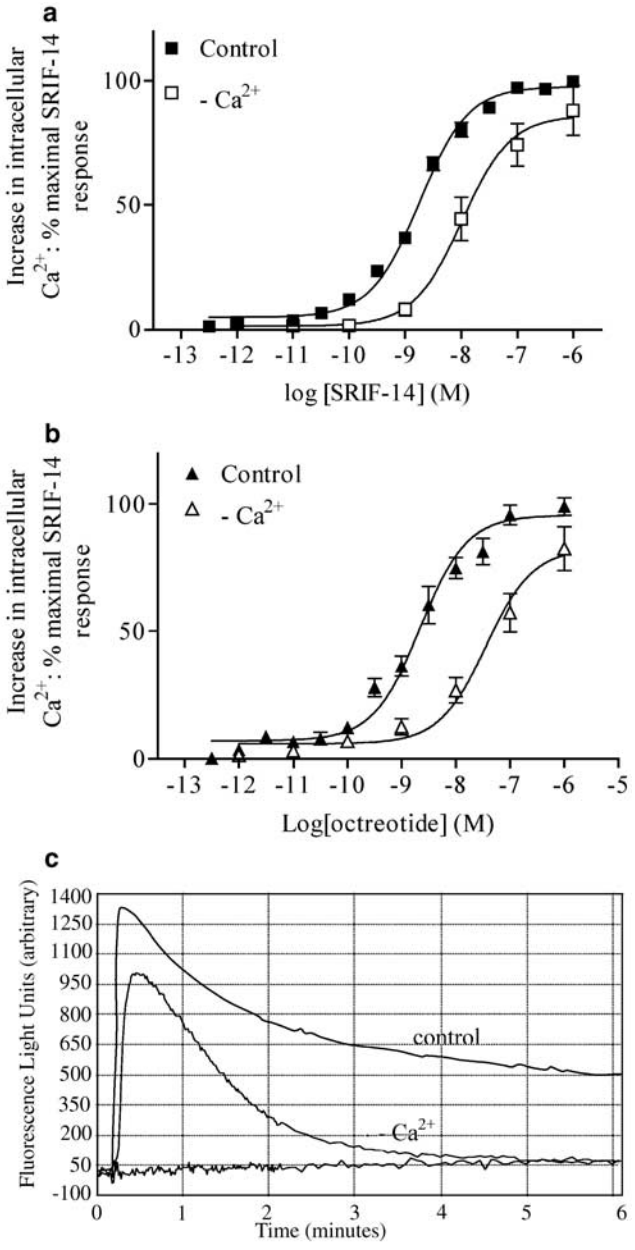

Figure 5 Effect of removing extracellular Ca^{2+} on SRIF-14/octreotide-stimulated intracellular Ca^{2+} modulation in CHO-K1 cells expressing human sst_2 receptors. Cells were incubated with Fluo-4 AM for 1 h. Plates were washed using buffer with or without Ca^{2+} and placed into a FLIPR™ where fluorescence intensity was determined before and after the addition of quadruplicates of SRIF-14/octreotide in the presence or absence of extracellular Ca^{2+}. EGTA (1 mM) was included in the 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±s.e.m. of all experiments ($n \geqslant 16$). Data in (c) is a representative trace of kinetics of 1 μM SRIF-14 in the presence and absence of extracellular calcium.

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

Thapsigargin (1 μM) 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±719.9 FLU, $n=3$, without thapsigargin, 6774.7±299.4 FLU, $n=3$, with thapsigargin, $P=0.135$); however, it abolished the effect of both SRIF-14 and octreotide on intracellular 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 (10.03×2.0 RLU, $n=3$, with thapsigargin compared to 19.9±2.8 RLU, $n=3$, without thapsigargin), there was no difference ($P \geqslant 0.46$) in maximum effect or potency of SRIF-14 and octreotide in the presence of thapsigargin (Figure 6c and d; E_{max}: 107.9±3.6 and 88.3±2.7% of maximal SRIF-14 response, pEC_{50}: 8.92±0.10 and 9.50±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 Ca^{2+} concentrations, extracellular acidification rates using microphysiometry, [^{35}S]GTPγS 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 Ca^{2+} using FLIPR II, and (ii) SRE-driven luciferase expression at human 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 sst_2 receptor, since the CHO-K1 cells used to express the receptor were also

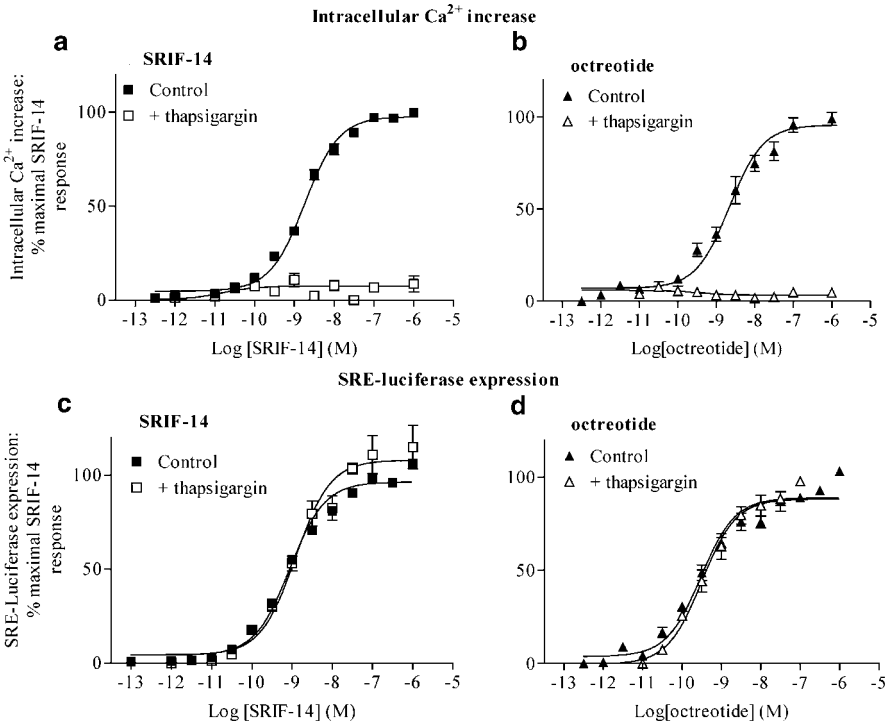

Figure 6 Effect of thapsigargin on SRIF-14/octreotide-stimulated intracellular Ca^{2+} modulation and SRE-luciferase reporter gene activity in CHO-K1 cells expressing human sst_2 receptors. Cells were incubated with Fluo-4 AM, in the presence or absence of thapsigargin (1 μM) for 1 h. Following washing, 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 ± s.e.m. of all experiments ($n \geqslant 4$).

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 Ca^{2+} *via* sst_2 receptors in recombinant cell lines including COS-7 (Akbar *et al*., 1994; Tomura *et al*., 1994), F_4C_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 (Rosskopf *et al*., 2003). In the majority of studies, the increase in intracellular 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; Rosskopf *et al*., 2003). Although, in cultured striatal astrocytes and rat AR42J pancreas cells, SRIF-induced rises in intracellular 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 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}S]GTPγS binding

in CCL39 cells expressing human sst_2 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^{2+} and luciferase expression.

Functional potency (pEC_{50}) in the two assays was lower than pK_d determined in binding. The reason for this may be due to differences in assay conditions (whole cells vs membranes, different Na^+ 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_2 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_i/G_o proteins (as we have shown previously, Nunn *et al*., 2002). In contrast, intracellular Ca^{2+} stimulation appears to be only partially mediated by G_i/G_o proteins. This is in agreement with previous findings that stimulation of phospholipase C, leading to an increase in intracellular Ca^{2+} by SRIF receptors, is only partially inhibited by pertussis toxin in COS-7 cells expressing human sst_2, sst_3 and sst_5 receptors (Akbar *et al*., 1994; Tomura *et al*., 1994), F_4C_1 cells expressing sst_2 receptors (Chen *et al*., 1997), CCL39 cells expressing sst_2, sst_3 and sst_5 (Siehler & Hoyer, 1999c) and in B lymphoblasts which predominantly express sst_{2A} receptors (Rosskopf *et al*., 2003). The pertussis toxin-insensitive part of the effect has been suggested to be due to stimulation of G_q (or members of the G_q/G_{11} family) by SRIF (Akbar *et al*., 1994; Tomura *et al*., 1994; Chen *et al*., 1997; Siehler & Hoyer, 1999c; Rosskopf *et al*., 2003), although this has never been explicitly proven. The potency and efficacy of SRIF-14 and octreotide to stimulate intracellular Ca^{2+} were reduced in the presence of pertussis toxin, which may show that coupling to G_i/G_o 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^{2+} in nonexcitable cells *via* both influx of extracellular Ca^{2+} 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; Rosskopf *et al*., 2003), the mechanism by which SRIF, *via* sst_2, produces an increase in intracellular Ca^{2+} 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^{2+} increase with thapsigargin suggests that release of Ca^{2+} from intracellular stores is the primary mechanism involved. However, removal of extracellular Ca^{2+} 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^{2+} are involved in the regulation of intracellular Ca^{2+} by SRIF, with the initial peak being mainly due to mobilisation of intracellular Ca^{2+} stores and the sustained plateau being the result of Ca^{2+} influx across the plasma membrane. This is in accordance with the accepted model of agonist-stimulated intracellular Ca^{2+} 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^{2+} influx is secondary to the mobilisation of intracellular Ca^{2+} and therefore is probably due to a capacitative entry mechanism (Berridge, 1997; Taylor & Broad, 1998). It has been shown that sst_2 expressed in COS-7 cells, sst_5 expressed in CHO-K1 cells and endogenous sst_{2A} in B lymphoblasts increase intracellular Ca^{2+} by mechanisms involving both intracellular and extracellular Ca^{2+} as reported here (Tomura *et al*., 1994; Wilkinson *et al*., 1997; Rosskopf *et al*., 2003). In contrast, in rat pancreas AR42J cells, SRIF-induced increase in intracellular Ca^{2+}, mediated by sst_2, occurs solely *via* influx from extracellular Ca^{2+} (Taylor, 1995).

The luciferase response is apparently independent of intracellular Ca^{2+} 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^{2+} on luciferase expression since the cells did not function properly without extracellular Ca^{2+} 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_2 receptor expressed in CHO-K1 cells. It shows that ligand binding to the sst_2 receptor can induce increases in both intracellular Ca^{2+} and luciferase expression. Comparison of the profiles of the two responses suggest that the sst_2 receptor is acting classically with parallel efficacy and potency profiles for luciferase expression and intracellular Ca^{2+}, although luciferase expression may show higher receptor reserve or better coupling, having higher potency than intracellular Ca^{2+} stimulation. The data show that when determined simultaneously, the effects of SRIF ligands on intracellular Ca^{2+} 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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References

AKBAR, M., OKAJIMA, F., TOMURA, H., MAJID, M.A., YAMADA, Y., SEINO, S. & KONDO, Y. (1994). Phospholipase C activation and Ca^{2+} mobilization by cloned human somatostatin receptor subtypes 1–5, in transfected COS-7 cells. *FEBS Lett.,* **348,** 192–196.

AKOPIAN, A., JOHNSON, J., GABRIEL, R., BRECHA, N. & WITKOVSKY, P. (2000). Somatostatin modulates voltage-gated K(+) and Ca(2+) currents in rod and cone photoreceptors of the salamander retina. *J. Neurosci.,* **20,** 929–936.

BERG, K.A., MAAYANI, S. & CLARKE, W.P. (1998a). Interactions between effectors linked to serotonin receptors. *Ann. N.Y. Acad. Sci.,* **861,** 111–120.

BERG, K.A., MAAYANI, S., GOLDFARB, J. & CLARKE, W.P. (1998b). Pleiotropic behavior of 5-HT2A and 5-HT2C receptor agonists. *Ann. N.Y. Acad. Sci.,* **861,** 104–110.

BERG, K.A., MAAYANI, S., GOLDFARB, J., SCARAMELLINI, C., LEFF, P. & CLARKE, W.P. (1998c). Effector pathway-dependent relative efficacy at serotonin type 2A and 2C receptors: evidence for agonist-directed trafficking of receptor stimulus. *Mol. Pharmacol.,* **54,** 94–104.

BERRIDGE, M.J. (1993). Inositol trisphosphate and calcium signalling. *Nature,* **361,** 315–325.

BERRIDGE, M.J. (1997). Elementary and global aspects of calcium signalling. *J. Physiol.,* **499,** 291–306.

BITO, H., MORI, M., SAKANAKA, C., TAKANO, T., HONDA, Z., GOTOH, Y., NISHIDA, E. & SHIMIZU, T. (1994). Functional coupling of SSTR4, a major hippocampal somatostatin receptor, to adenylate cyclase inhibition, arachidonate release, and activation of the mitogen-activated protein kinase cascade. *J. Biol. Chem.,* **269,** 12722–12730.

CARRUTHERS, A.M., WARNER, A.J., MICHEL, A.D., FENIUK, W. & HUMPHREY, P.P.A. (1999). Activation of adenylate cyclase by human recombinant sst5 receptors expressed in CHO-K1 cells and involvement of G.alpha.s proteins. *Br. J. Pharmacol.,* **126,** 1221–1229.

CHAI, J. & TARNAWSKI, A.S. (2002). Serum response factor: discovery, biochemistry, biological roles and implications for tissue injury healing. *J. Physiol. Pharmacol.,* **53,** 147–157.

CHEN, L., FITZPATRICK, V.D., VANDLEN, R.L. & TASHJIAN JR, A.H. (1997). Both overlapping and distinct signaling pathways for somatostatin receptor subtypes SSTR1 and SSTR2 in pituitary cells. *J. Biol. Chem.,* **272,** 18666–18672.

CHEN, L. & TASHJIAN, A. (1994). *Progress of the 76th Annual Meeting of The Endocrine Society. Abstr,* **1555**, p. 589.

DE LEAN, A. (1979). SCTFIT: a computer program for simultaneous analysis of saturation and competition curves. Howard Hughes medical institute, Duke University medical center, Durham, North Carolina.

FEUERBACH, D., FEHLMANN, D., NUNN, C., SIEHLER, S., LANGENEGGER, D., BOUHELAL, R., SEUWEN, K. & HOYER, D. (2000). Cloning, expression and pharmacological characterization of the mouse somatostatin sst5 receptor. *Neuropharmacology,* **39,** 1451–1462.

FLORIO, T. & SCHETTINI, G. (1996). Multiple intracellular effectors modulate physiological functions of the cloned somatostatin receptors. *J. Mol. Endocrinol.,* **17,** 89–100.

GILLE, H., KORTENJANN, M., THOMAE, O., MOOMAW, C., SLAUGHTER, C., COBB, M.H. & SHAW, P.E. (1995). ERK phosphorylation potentiates Elk-1-mediated ternary complex formation and transactivation. *EMBO J.,* **14,** 951–962.

HANNON, J.P., NUNN, C., STOLZ, B., BRUNS, C., WECKBECKER, G., LEWIS, I., TROXLER, T., HURTH, K. & HOYER, D. (2002). Drug design at peptide receptors: somatostatin receptor ligands. *J. Mol. Neurosci.,* **18,** 15–27.

HAY, B.A., COLE, B.M., DICAPUA, F.M., KIRK, G.W., MURRAY, M.C., NARDONE, R.A., PELLETIER, D.J., RICKETTS, A.P., ROBERTSON, A.S. & SIEGEL, T.W. (2001a). Small molecule somatostatin receptor subtype-2 antagonists. *Bioorg. Med. Chem. Lett.,* **11,** 2731–2734.

HAY, B.A., COLE, B.M. & RICKETTS, A.P. (2001b). Somatostatin antagonists and agonists that act at the SST subtype 2 receptor. European Patent Application, EP 1 086 947 A1.

HILL, C.S. & TREISMAN, R. (1995). Differential activation of c-fos promoter elements by serum, lysophosphatidic acid, G proteins and polypeptide growth factors. *EMBO J.,* **14,** 5037–5047.

HOYER, D., BELL, G.I., BERELOWITZ, M., EPELBAUM, J., FENIUK, W., HUMPHREY, P.P., O'CARROLL, A.M., PATEL, Y.C., SCHONBRUNN, A., TAYLOR, J.E. & REISINE, T. (1995). Classification and nomenclature of somatostatin receptors. *Trends Pharmacol. Sci.,* **16,** 86–88.

KAMIYA, Y., OHMURA, E., ARAI, M., FUJII, T., HAYAKAWA, F., ITO, J., KAWAGUCHI, M., TSUSHIMA, T. & SAKUMA, N. (1993). Effect of somatostatin and its analogue on proliferation of human epidermoid carcinoma cells *in vitro*. *Biochem. Biophys. Res. Commun.,* **191,** 302–307.

KOMATSUZAKI, K., MURAYAMA, Y., GIAMBARELLA, U., OGATA, E., SEINO, S. & NISHIMOTO, I. (1997). A novel system that reports the G-proteins linked to a given receptor: a study of type3 somatostatin receptor. *FEBS Lett.,* **406,** 165–170.

LACHOWICZ, A., PAWLIKOWSKI, M. & OCHEDALSKI, T. (1994). Somatostatin-14 increases the inositol-1,4,5-trisphosphate content in various areas of the brain. *Biochem. Biophys. Res. Commun.,* **203,** 379–384.

LACHOWICZ, A., STEPIEN, H., KUNERT-RADEK, J. & PAWLIKOWSKI, M. (1992). Effects of somatostatin on inositol-1,4,5-trisphosphate content in mouse spleen lymphocytes. *Comp. Biochem. Physiol.,* **101,** 661–664.

LEFF, P. & SCARAMELLINI, C. (1998). Promiscuity, pre-coupling and instability. *Trends Pharmacol. Sci.,* **19,** 13.

MALM, D., GIAEVER, A., VONEN, B., BURHOL, P.G. & FLORHOLMEN, J. (1991). Somatostatin inhibition of phospholipase C activity in isolated rat pancreatic islets. *Acta Physiol. Scand.,* **143,** 413–419.

MARIN, P., DELUMEAU, J.C., TENCE, M., CORDIER, J., GLOWINSKI, J. & PREMONT, J. (1991). Somatostatin potentiates the alpha 1-adrenergic activation of phospholipase C in striatal astrocytes through a mechanism involving arachidonic acid and glutamate. *Proc. Natl. Acad. Sci. U.S.A.,* **88,** 9016–9020.

MEYERHOF, W. (1998). The elucidation of somatostatin receptor functions: a current view. *Rev. Physiol. Biochem. Pharmacol.,* **133,** 55–105.

MURTHY, K.S., COY, D.H. & MAKHLOUF, G.M. (1996). Somatostatin receptor-mediated signaling in smooth muscle. Activation of phospholipase C-beta3 by Gbetagamma and inhibition of adenylyl cyclase by Galphai1 and Galphao. *J. Biol. Chem.,* **271,** 23458–23463.

NUNN, C., FEUERBACH, D., LIN, X., PETER, R. & HOYER, D. (2002). Pharmacological characterisation of the goldfish somatostatin sst5 receptor. *Eur. J. Pharmacol.,* **436,** 173–186.

NUNN, C., LANGENEGGER, D., HURTH, K., SCHMIDT, K., FEHLMANN, D. & HOYER, D. (2003a). Agonist properties of putative small-molecule somatostatin sst(2) receptor-selective antagonists. *Eur. J. Pharmacol.,* **465,** 211–218.

NUNN, C., RUEPING, M., LANGENEGGER, D., SCHUEPBACH, E., KIMMERLIN, T., MICUCH, P., HURTH, K., SEEBACH, D. & HOYER, D. (2003b). beta(2)/beta(3)-di- and alpha/beta(3)-tetrapeptide derivatives as potent agonists at somatostatin sst(4) receptors. *Naunyn Schmiedebergs Arch. Pharmacol.,* **367,** 95–103.

NUNN, C., SCHOEFFTER, P., LANGENEGGER, D. & HOYER, D. (2003c). Functional characterisation of the putative somatostatin sst2 receptor antagonist CYN 154806. *Naunyn Schmiedebergs Arch. Pharmacol.,* **367,** 1–9.

PATEL, Y.C., GREENWOOD, M.T., WARSZYNSKA, A., PANETTA, R. & SRIKANT, C.B. (1994). All five cloned human somatostatin receptors (hSSTR1-5) are functionally coupled to adenylyl cyclase. *Biochem. Biophys. Res. Commun.,* **198,** 605–612.

PATEL, Y.C. (1999). Somatostatin and its receptor family. *Front. Neuroendocrinol.,* **20,** 157–198.

PATEL, Y.C. & SRIKANT, C.B. (1997). Somatostatin receptors. *Trends Endocrinol. Metab.,* **8,** 398–405.

PRICE, M.A., ROGERS, A.E. & TREISMAN, R. (1995). Comparative analysis of the ternary complex factors Elk-1, SAP-1a and SAP-2 (ERP/NET). *EMBO J.,* **14,** 2589–2601.

RAYNOR, K., MURPHY, W.A., COY, D.H., TAYLOR, J.E., MOREAU, J.P., YASUDA, K., BELL, G.I. & REISINE, T. (1993). Cloned somatostatin receptors: identification of subtype-selective peptides and demonstration of high affinity binding of linear peptides. *Mol. Pharmacol.,* **43,** 838–844.

ROMOSER, V.A., GRAVES, T.K., WU, D., JIANG, H. & HINKLE, P.M. (2001). Calcium responses to thyrotropin-releasing hormone, gonadotropin-releasing hormone and somatostatin in phospholipase css3 knockout mice. *Mol. Endocrinol.,* **15,** 125–135.

ROSSKOPF, D., SCHURKS, M., MANTHEY, I., JOISTEN, M., BUSCH, S. & SIFFERT, W. (2003). Signal transduction of somatostatin in human B lymphoblasts. *Am. J. Physiol.,* **284,** C179–C190.

SCARAMELLINI, C. & LEFF, P. (1998). A three-state receptor model: predictions of multiple agonist pharmacology for the same receptor type. *Ann. N.Y. Acad. Sci.,* **861,** 97–103.

SCHOEFFTER, P., PEREZ, J., LANGENEGGER, D., SCHUEPBACH, E., BOBIRNAC, I., LUEBBERT, H., BRUNS, C. & HOYER, D. (1995). Characterization and distribution of somatostatin SS-1 and SRIF-1 binding sites in rat brain: identity with SSTR-2 receptors. *Eur. J. Pharmacol.,* **289,** 163–173.

SIEHLER, S. & HOYER, D. (1999a). Characterization of human recombinant somatostatin receptors. 2. Modulation of GTP.gamma.S binding. *Naunyn Schmiedebergs Arch. Pharmacol.,* **360,** 500–509.

SIEHLER, S. & HOYER, D. (1999b). Characterization of human recombinant somatostatin receptors. 3. Modulation of adenylate cyclase activity. *Naunyn Schmiedebergs Arch. Pharmacol.,* **360,** 510–521.

SIEHLER, S. & HOYER, D. (1999c). Characterization of human recombinant somatostatin receptors. 4. Modulation of phospholipase C activity. *Naunyn Schmiedebergs Arch. Pharmacol.,* **360,** 522–532.

SIEHLER, S., SEUWEN, K. & HOYER, D. (1999). Characterization of human recombinant somatostatin receptors. 1. Radioligand binding studies. *Naunyn Schmiedebergs Arch. Pharmacol.,* **360,** 488–499.

SMART, D., JERMAN, J.C., BROUGH, S.J., RUSHTON, S.L., MURDOCK, P.R., JEWITT, F., ELSHOURBAGY, N.A., ELLIS, C.E., MIDDLEMISS, D.N. & BROWN, F. (1999). Characterization of recombinant human orexin receptor pharmacology in a Chinese hamster ovary cell-line using FLIPR. *Br. J. Pharmacol.,* **128,** 1–3.

TAYLOR, C.W. & BROAD, L.M. (1998). Pharmacological analysis of intracellular Ca^{2+} signalling: problems and pitfalls. *Trends Pharmacol. Sci.,* **19,** 370–375.

TAYLOR, J.E. (1995). Somatostatin (SSTR2) receptors mediate phospholipase C-independent Ca^{2+} mobilization in rat AR42J pancreas cells. *Biochem. Biophys. Res. Commun.,* **214,** 81–85.

TOMURA, H., OKAJIMA, F., AKBAR, M., ABDUL, M., SHO, K. & KONDO, Y. (1994). Transfected human somatostatin receptor type 2, SSTR2, not only inhibits adenylate cyclase but also stimulates phospholipase C and Ca^{2+} mobilization. *Biochem. Biophys. Res. Commun.,* **200,** 986–992.

TREISMAN, R. (1994). Ternary complex factors: growth factor regulated transcriptional activators. *Curr. Opin. Genet. Dev.,* **4,** 96–101.

TREISMAN, R. (1995). Journey to the surface of the cell: Fos regulation and the SRE. *EMBO J.,* **14,** 4905–4913.

WHITMARSH, A.J., SHORE, P., SHARROCKS, A.D. & DAVIS, R.J. (1995). Integration of MAP kinase signal transduction pathways at the serum response element. *Science,* **269,** 403–407.

WHITMARSH, A.J., YANG, S.H., SU, M.S., SHARROCKS, A.D. & DAVIS, R.J. (1997). Role of p38 and JNK mitogen-activated protein kinases in the activation of ternary complex factors. *Mol. Cell. Biol.,* **17,** 2360–2371.

WILKINSON, G.F., FENIUK, W. & HUMPHREY, P.P.A. (1997). Homologous and heterologous desensitization of somatostatin-induced increases in intracellular Ca^{2+} and inositol 1,4,5-trisphosphate in CHO-K1 cells expressing human recombinant somatostatin sst5 receptors. *Eur. J. Pharmacol.,* **340,** 277–285.

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