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Pharmacological characterisation of the goldfish somatostatin sst_5 receptor

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Abstract

Somatostatin (somatotropin release inhibiting factor, SRIF), exerts its effects via specific G protein coupled receptors of which five subtypes have been cloned (sst_{1-5}). Recently, SRIF receptors have also been cloned from fish tissues. In this study, goldfish sst_5 receptors ($gfsst_5$) were expressed and characterised in the Chinese hamster lung fibroblast cell line, that harbours the luciferase reporter gene driven by the serum responsive element (CCL39-SRE-Luci). The agonist radioligands [^{125}I]-LTT-SRIF-28 ([Leu^8, DTrp^{22}, ^{125}I-Tyr^{25}]SRIF-28) and [^{125}I][Tyr^{10}]cortistatin-14 labelled similar receptor densities with high affinity and in a saturable manner (pK_d: 9.99–9.71; B_{max}: 300–350 fmol mg^{-1}). 5′ -Guanylyl-imidodiphosphate inhibited radioligand binding to some degree (38.5–57.9%). In competition binding studies, the pharmacological profile of SRIF binding sites defined with [^{125}I]LTT-SRIF-28 and [^{125}I][Tyr^{10}]cortistatin-14 correlated significantly ($r^2 = 0.97$, $n = 20$). Pharmacological profiles of human and mouse sst_5 receptors expressed in CCL39 cells correlated markedly less with those of the $gfsst_5$ profile ($r^2 = 0.52–0.78$, $n \geq 16$). Functional expression of the $gfsst_5$ receptor was examined by measurement of agonist-induced luciferase expression and stimulation of [^{35}S]GTPγS ([^{35}S]guanosine 5′ -*O*-(3-thiotriphosphate) binding. Profiles were similar to those achieved in radioligand binding studies ($r^2 = 0.81–0.93$, $n = 20$), although relative potency (pEC_{50}) was reduced compared to pK_d values. Relative efficacy profiles of luciferase expression and [^{35}S]GTPγS binding, were rather divergent ($r^2 = 0.48$, $n = 20$) with peptides showing full agonism at one pathway and absence of agonism at the other. BIM 23056 (D-Phe-Phe-Tyr-D-Trp-Lys-Val-Phe-D-Nal-NH_2) acted as an antagonist on the effects of SRIF-14 (p$K_B = 6.74 \pm 0.23$) on stimulation of [^{35}S]GTPγS binding. Pertussis toxin abolished the effect of SRIF-14 on luciferase expression and [^{35}S]GTPγS binding suggesting coupling of the receptor to G_i/G_o proteins. In summary, the present studies demonstrate that the $gfsst_5$ receptor has a similar pharmacological profile and transductional properties to mammalian sst_5 receptors. The difference in efficacy profiles defined using different functional assays suggests numerous, agonist specific, conformational receptor states, and/or ligand-dependent receptor trafficking.

Keywords: (SRIF) Somatostatin; Somatostatin sst_5 receptor, goldfish; Luciferase; (SRE) Serum responsive element; ([^{35}S]GTPγS); Receptor trafficking

1. Introduction

Somatostatin or SRIF (somatotropin release inhibiting factor) is a hormone/neuropeptide which is found at high levels in the central and peripheral nervous system, and a number of peripheral tissues. It is involved in the regulation of multiple physiological processes, including endocrine and exocrine secretions, neurotransmission, neuromodulation of transmitter release, smooth muscle motility and cell proliferation, especially in tumours (Reichlin, 1983; Patel, 1997).

In mammals, SRIF is found in two forms—SRIF-14, and the N-terminally extended SRIF-28. These two peptides are produced from a single gene which encodes preprosomatostatin (PSS); this is differentially cleaved to either SRIF-14 or SRIF-28 (Pradayrol et al., 1980; Patel and O'Neil, 1988; Patel and Galanopoulou, 1995). SRIF-14 is highly conserved throughout evolution, being found in all mammalian species studied and in representatives from all vertebrate classes including birds, amphibians, reptiles and fish (Lin et al., 2000b). Although rare, variants of SRIF-14 are also found in some species including [Ser^{12}]SRIF-14 in Sea Lamprey (Andrews et al., 1988), [Ser^5]SRIF-14 in Pacific Ratfish (Conlon, 1990), [Pro^2,Met^{13}]SRIF-14 in European Green Frog (Vaudry et al., 1992) and [Pro^2]SRIF-14 in Russian Sturgeon, African Lungfish and Goldfish (Nishii et al., 1995; Lin et al., 1999b; Trabucchi et al., 1999).

Recently, it has been shown that SRIF is part of a multigene family. A second SRIF precursor gene (PSS-II) has been identified in teleost fish, that is processed to SRIF

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peptides of 28, 25, or 14 amino acids in length with [Tyr^7,Gly^{10}]SRIF-14 (or a variant of this depending on the species) at their C terminus (Conlon et al., 1987; Zupanc et al., 1999; Lin et al., 2000b). In frog brain, a second PSS gene has been identified which is processed to [Pro^2,Met^{13}]SRIF-14 (Vaudry et al., 1992). A prepropeptide cDNA with structural similarity to that of preprosomatostatin has also been cloned in mammals. This is cleaved to produce cortistatin which is found in rat, mouse and human brain in various forms and has a 14 amino acid peptide at its C-terminus with 11 amino acids identical to SRIF-14 (De Lecea et al., 1996; 1997; Fukusumi et al., 1997). Goldfish have three distinct SRIF precursor genes. These are termed PSS-I, PSS-II and PSS-III and are processed to SRIF-14, goldfish SRIF-28 which has [Glu^1, Tyr^7, Gly^{10}]SRIF-14 at its C terminus, and [Pro^2]SRIF-14, respectively (Lin et al., 1999b, 2000b).

SRIF produces its effects by binding to high affinity membrane bound G protein-coupled receptors (Schonbrunn and Tashjian, 1978; Jakobs et al., 1983; Bell and Reisine, 1993) of which five subtypes (sst_{1-5}) have been identified from several mammalian species (Bell and Reisine, 1993; Hoyer et al., 1994, 1995). The receptors display a subtype-specific distribution pattern in brain and peripheral tissues (Bell and Reisine, 1993; Patel, 1997; Selmer et al., 2000). All five receptor subtypes bind SRIF-14 and SRIF-28 with high affinity, but differ in their ability to bind the short-chain synthetic analogues octreotide, seglitide and somatuline, producing two distinct subclasses according to pharmacology and structure: $sst_{2,}$ $sst_{3\ and}$ sst_5, with high affinity for the short analogues and $sst_{1\ and\ 4}$, with low affinity (Hoyer et al., 1995).

In goldfish, cDNAs for two receptors corresponding to mammalian sst_1, one corresponding to mammalian sst_2 and one to mammalian sst_5 have been cloned (Lin et al., 1999a, 2000a; submitted). The sst_1 receptors (termed sst_{1A} and sst_{1B}) have 98% homology in their amino acid sequences and are thought to be the product of duplicate genes rather than spliced variants (Lin et al., 1999a). An sst_3 subtype ($fsst_3$) has been isolated in an electric fish *Apteronotus albifrons* (Siehler et al., 1999b; Zupanc et al., 1999).

In the present study, the newly cloned goldfish sst_5 ($gfsst_5$) receptor (Lin et al., 2002, in press) was expressed in Chinese hamster lung fibroblast cells (CCL39), and its pharmacological features examined and compared to those of human and mouse sst_5 using radioligand binding studies, and stimulation of [^{35}S]guanosine 5′ -*O*-(3-thiotriphosphate ([^{35}S]GTPγS) binding and coupling via the serum responsive element to luciferase expression.

2. Methods

2.1. Cell culture

CCL39-SRE-Luci cells (established line of Chinese hamster lung fibroblasts; American Type Culture Collection), were cultured in a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM; Seromed, Biochrom, Berlin, Germany: 3.7 g l^{-1} $NaHCO_3$, 1.0 g l^{-1} D-glucose, with stable glutamine) and Ham's F-12 Nutrient Mixture (Seromed: 1.176 g l^{-1} $NaHCO_3$, with stable glutamine), supplemented with 10% (v/v) foetal bovine serum (Gibco BRL) and Hygromycin B (100 μg ml^{-1}) (Calbiochem, La Jolla, CA, USA) at 37 °C, 5% CO_2 and 95% relative humidity. The cells were passaged every 2 days by washing with phosphate-buffered saline (PBS; Gibco BRL) and brief incubation with trypsin (0.5 mg ml^{-1})/EDTA (0.2 mg ml^{-1}) (Gibco BRL). For storage, the cells were resuspended in medium containing 10% dimethyl sulfoxide and 20% foetal bovine serum, and frozen in liquid nitrogen.

2.2. Stable transfection

CCL39-SRE-Luci cells were used for stable expression of the goldfish sst_5 receptor. Cells were split 1 day prior to transfection for logarithmic growth. Cells at 40–80% confluence in 6-cm Petri dishes were incubated at 37 °C, 5% CO_2, 95% relative humidity with growth medium containing 2.5–10 μg DNA (pcDNA3.1(−)-$gfsst_5$; Dr. X. Lin, University of Alberta, Edmonton, Alberta, Canada), and 2% superfect reagent (Qiagen, Basel, Switzerland). After 3 h, the cells were washed four times with PBS and incubated in normal growth medium. The day after the transfection, the cells were split to 10-cm Petri dishes and the antibiotic G418 (geneticin sulphate, Gibco BRL) was added to the cell culture medium (0.4 mg ml^{-1}) for selection of SRIF receptor-expressing cells. Receptor expression of single cell-derived colonies was tested by radioligand binding. Transfected cells were permanently cultured in G418-containing medium (100 μg ml^{-1}).

2.3. Radioligand binding assay

For crude cell membrane preparations, CCL39-SRE-Luci-$gfsst_5$ cells were washed with 10 mM HEPES, 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. The cells were resuspended in binding assay buffer (10 mM HEPES, pH 7.5, 0.5% (w/v) bovine serum albumin) by homogenisation with a Polytron homogeniser at 50 Hz for 20 s.

In competition experiments, 150 μl of cell homogenate was incubated with 50 μl of [^{125}I]LTT-SRIF-28 ([Leu^8, DTrp^{22}, ^{125}I-Tyr^{25}]SRIF-28) or [^{125}I][Tyr^{10}]cortistatin-14 (2175 Ci $mmol^{-1}$, 25–35 pM final concentration), in binding assay buffer containing $MgCl_2$ (5 mM) and the protease inhibitor bacitracin (5 μg ml^{-1}), and either 50 μl assay buffer (total binding); SRIF-14 at a concentration of 10 μM (non-specific binding); or various peptide/5′ -Guanylyl-imidodiphosphate (GppNHp) concentrations. Experiments were conducted in triplicate. Incubation was terminated after 1 h at room temperature by vacuum filtration through glass fibre filters pre-soaked in 0.3% polyethyleneimine. The filters

were washed three times with ice-cold 10 mM Tris–HCl buffer containing 154 mM NaCl, pH 7.4 and dried. Bound radioactivity was measured in a γ-counter using scintillation liquid (80% counting efficiency). Data were analysed by non-linear regression curve fitting with the computer program SCTFIT (De Lean, 1979).

In saturation experiments, 150 μl of cell homogenate were incubated with 50 μl of eight different concentrations (approximately 25–300 pM) of [^{125}I]LTT-SRIF-28 or [^{125}I][Tyr^{10}]cortistatin-14, and 50 μl of assay buffer (total binding) or 1 μM SRIF-14 (non-specific binding). Experiments were conducted in triplicate. If GppNHp (10 μM final concentration) was used, it was included in each well. Data was analysed using the computer program Graph Pad Prism. Protein concentration was determined according to Bradford (1976) by means of the BioRad Protein Assay Kit using bovine serum albumin as a standard.

2.4. Agonist-stimulated [^{35}S]GTPγS binding assay

For microsome preparations, CCL39-SRE-Luci-gfsst$_5$ cells were washed with 10 mM HEPES, 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 preparations were resuspended in 10 mM HEPES, pH 7.5 by Polytron homogenisation at 50 Hz for 20 s, and centrifuged at 4 °C for 30 min at 15000 × *g*. The microsome pellets were resuspended in assay buffer (10 mM HEPES pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA pH 7.5, 10 μg ml^{-1} bacitracin), and either stored at −80 °C or directly used. In experiments with pertussis toxin, the cells were incubated for 18 h with 100 ng ml^{-1} pertussis toxin before harvesting.

One hundred microliters of the microsome preparation (1×10^5 cells) was incubated in 96-well plates (Dynatech Laboratories) with [^{35}S]GTPγS (200 pM; final concentration) in assay buffer containing 1 μM GDP and triplicates of either: assay buffer (basal); 10 μM GTPγS (non-specific binding (NS)); 10 μM SRIF-14 (positive agonist control); or SRIF ligands at various concentrations. After 5 min preincubation, 50 μl wheatgerm agglutinin scintillation proximity assay-beads were added (1.5 mg beads per well; beads in 50 mM Tris pH 7.4, 0.1% sodium azide, and diluted 1:3 in assay buffer), the plates sealed, incubated for 30 min at room temperature, and centrifuged for 10 min at 1000 × *g*. [^{35}S]GTPγS bound to a G-protein/receptor-complex, and thereby to the wheatgerm agglutinin scintillation proximity assay beads which stimulated them to emit light, which was measured in a β-scintillation counter (Packard TopCount). Activation (% of basal GTPγS binding (=100%)) was calculated as:

$$\text{Activation (\%)} = \frac{\text{Experimental} - \text{NS}}{\text{Basal} - \text{NS}} \times 100$$

Stimulatory concentration–response curves were analysed by non-linear regression curve fitting using the programme Graph Pad Prism. pK_B values were determined using the Schild–Gaddum equation. The response to SRIF-14 (positive agonist control) was designated as 100% response and the E_{max} of all other ligands were calculated as a percentage of the SRIF-14 response.

2.5. Luciferase assay

CCL39-SRE-Luci-gfsst$_5$ cells were seeded at 60,000 cells well^{-1} in 96 well plates. After 24 h, the medium was removed and the cells were washed twice with PBS and serum-deprived for 24 h in HEPES-buffered salt solution (HBSS: 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), containing 1% bovine serum albumin, pH 7.4. The cells were treated for 5 h at 37 °C, 5% CO_2, 95% relative humidity with HBSS 0.1% bovine serum albumin alone (background) or containing various concentrations of SRIF ligands in triplicate. 10% foetal bovine serum was used as a positive control to show the SRE-Luci construct was functioning in the cells. Where pertussis toxin (100 ng ml^{-1}) was used it was added to the cells during serum deprivation 18 or 3 h before treatment with SRIF-14. Cells were lysed in 25 μl lysis buffer (25 mM Tris–Phosphate pH 7.8, 2 mM DL-dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol, 1% triton-X), and after 15 min luciferase activity was measured using a Luminoskan luminometer by injection of 50 μl luciferase reaction buffer (20 mM Tricine, 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). Measuring parameters: lag time 0 s, total integration time 5 s. Activation (% increase over basal) was calculated as:

$$\text{Activation (\%)} = \frac{\text{Experimental} - \text{background}}{\text{background}} \times 100$$

Stimulatory concentration–response curves were analysed by non-linear regression curve fitting using the programme Graph Pad Prism. The response to SRIF-14 was designated as 100% response and the E_{max} of all other ligands were calculated as a percentage of the SRIF-14 response.

2.6. Ligands

BIM 23014 (lanreotide; somatuline®; D-Nal-c[Cys-Tyr-D-Trp-Lys-Val-Cys]-Thr-NH_2), BIM 23030 (c[Mpr-Tyr-D-Trp-Lys-Val-Cys]-D-Phe-NH_2), BIM 23052 (D-Phe-Phe-Phe-D-Trp-Lys-Thr-Phe-Thr-NH_2), BIM 23056 (D-Phe-Phe-Tyr-D-Trp-Lys-Val-Phe-D-Nal-NH_2), CGP 23996 (c[Asu-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Tyr-Thr-Ser]), Mouse/Rat cortistatin-14 (Pro-c[Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Ser-Ser-Cys]-Lys), [Tyr^{10}]cortistatin-14 (Pro-c[Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Tyr-Ser-Ser-Cys]-Lys), Human cortistatin-17 (Asp-Arg-Met-Pro-c[Cys-Arg-Asp-Phe-Phe-Trp-Lys-Thr-Phe-Ser-Ser-Cys]-Lys), Cycloanta-

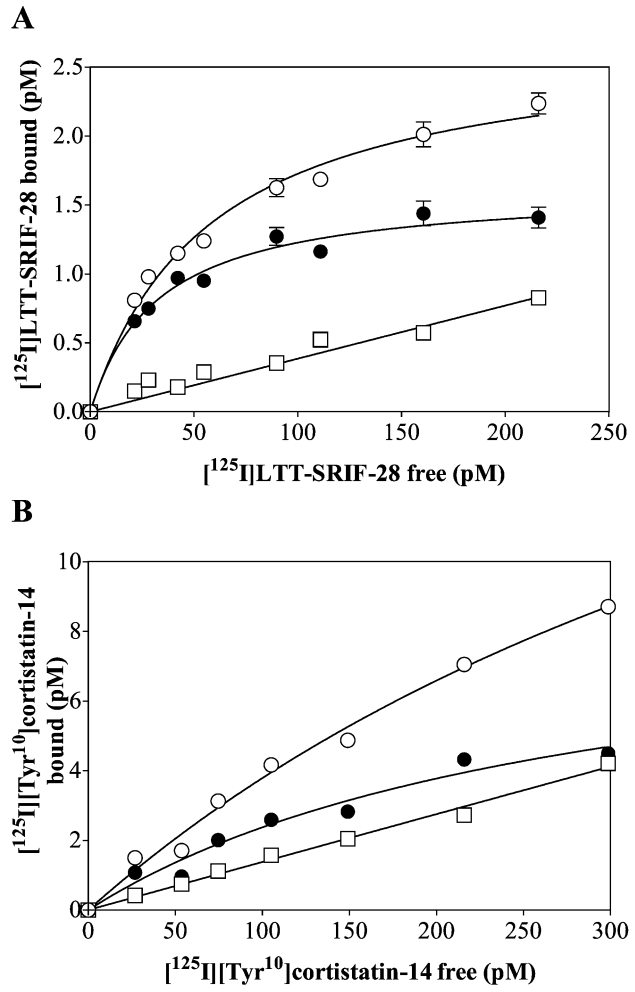

Fig. 1. Saturation binding of (A) [^{125}I]LTT-SRIF-28 and (B) [^{125}I][Tyr^{10}]cortistatin-14 to membranes prepared from CCL39-SRE-Luci cells stably expressing goldfish sst_5 receptors. Crude membrane preparations were incubated for 1 h at 22 °C with radioligand (25–300 pM) in the presence (□ non-specific) or absence (○ total) of SRIF-14 (10 μM) in triplicate to determine specific binding (●). The plots depict amount of radioligand bound versus free radioligand concentration (pM) and represent one example of four different experiments.

gonist (SA; c[Aha-Phe-D-Trp-Lys-Thr(Bzl)]), L362,855 (c[Aha-Phe-Trp-D-Trp-Lys-Thr-Phe]), Octreotide (SMS 201-995, Sandostatin®; D-Phe-c[Cys-Phe-D-Trp-Lys-Thr-Cys]-Thr-OH), [Tyr^3]octreotide (SDZ 204-090; D-Phe-c[Cys-Tyr-D-Trp-Lys-Thr-Cys]-Thr-OH), RC160 (vapreotide; octastatin; D-Phe-c[Cys-Tyr-D-Trp-Lys-Val-Cys]-Trp-NH_2), Seglitide (MK678; c[*N*-Met-Ala-Tyr-D-Trp-Lys-Val-Phe]), SRIF-14 (Ala-Gly-c[Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys]-OH), Goldfish, [Pro^2]SRIF-14 (Ala-Pro-c[Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-

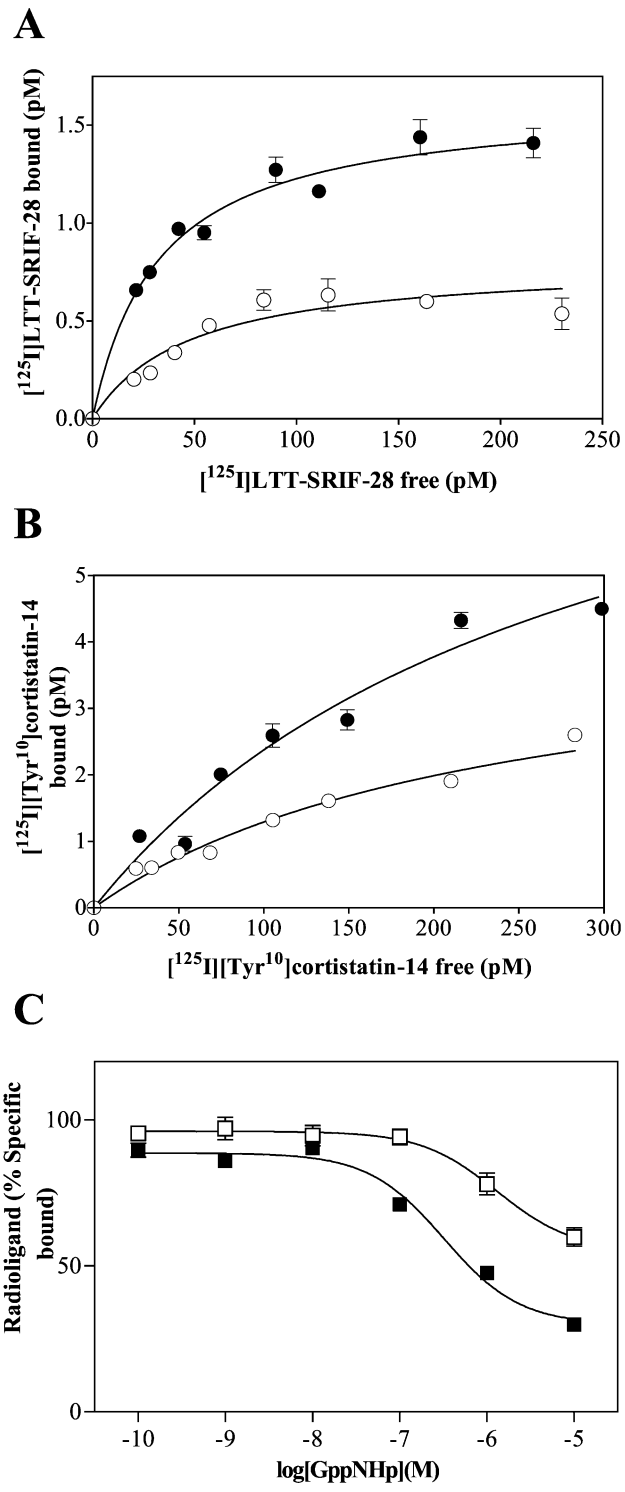

Fig. 2. Guanine nucleotide sensitivity of [^{125}I]LTT-SRIF-28 and [^{125}I][Tyr^{10}]cortistatin-14 binding to membranes prepared from CCL39-SRE-Luci cells stably expressing goldfish sst_5 receptors. (A and B) Crude membrane preparations were incubated for 1 h at 22 °C with radioligand (25–300 pM) and GppNHp (10 μM) in the presence (non-specific) or absence (total) of SRIF-14 (10 μM) in triplicate to determine specific binding. The plots show specific binding and depict amount of radioligand bound versus free radioligand concentration (pM) in the presence (○) or absence (●) of GppNHp. One example of four different experiments is represented. (C) Crude membrane preparation were incubated for 1 h at 22 °C with [^{125}I]LTT-SRIF-28 (□) or [^{125}I][Tyr^{10}]cortistatin-14 (■) (25–35 pM) and the indicated concentrations of GppNHp. Data is expressed as percentage specific binding. The plots represent one example of three experiments.

Table 1
Competition binding profiles of SRIF ligands with [^{125}I]LTT-SRIF-28 and [^{125}I][Tyr^{10}]cortistatin-14 at goldfish sst$_5$ receptors expressed in CCL39-SRE-Luci cells

	[^{125}I]LTT-SRIF-28	[^{125}I][Tyr^{10}]cortistatin-14
LTT-SRIF-28	10.00 ± 0.15	9.90 ± 0.08
SRIF-28	9.88 ± 0.19	9.67 ± 0.010
GF SRIF-28	9.81 ± 0.08	9.50 ± 0.10
SRIF-25	9.68 ± 0.09	9.71 ± 0.13
SRIF-14	9.19 ± 0.05	9.32 ± 0.19
GF SRIF-14	8.95 ± 0.13	8.93 ± 0.20
Cortistatin-17	8.76 ± 0.10	9.19 ± 0.03
RC 160	8.76 ± 0.47	8.84 ± 0.32
BIM 23052	8.71 ± 0.10	8.81 ± 0.11
[Tyr^{10}]Cortistatin-14	8.47 ± 0.15	8.78 ± 0.11
Cortistatin-14	8.42 ± 0.13	8.42 ± 0.15
BIM 23014	8.16 ± 0.08	7.97 ± 0.35
[Tyr^{3}]Octreotide	7.43 ± 0.10	7.71 ± 0.09
CGP 23996	7.32 ± 0.11	7.60 ± 0.10
Octreotide	7.19 ± 0.12	7.20 ± 0.17
Seglitide	7.02 ± 0.13	7.31 ± 0.02
BIM 23056	6.81 ± 0.08	6.90 ± 0.08
L362,855	6.68 ± 0.13	7.15 ± 0.10
Cycloantagonist SA	6.00 ± 0.07	6.10 ± 0.05
BIM 23030	5.85 ± 0.14	6.18 ± 0.05

The data are expressed as pK_d values (− log mol l^{-1}) ± S.E.M. of at least three determinations.

Cys]-OH), SRIF-25 (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), 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), Goldfish SRIF-28 (Ser-Ala-Glu-Ser-Ser-Asn-Gln-Leu-Pro-Thr-Arg-Val-Arg-Lys-Glu-Gly-c[Cys-Lys-Asn-Phe-Tyr-Trp-Lys-Gly-Phe-Thr-Ser-Cys]-OH), LTT-SRIF-28 ([Leu^{8},D-Trp^{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-D-Trp-Lys-Thr-Tyr-Thr-Ser-Cys]-OH), [^{125}I][Tyr^{10}]cortistatin-14 (Pro-c[Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-(^{125}I-Tyr)-Ser-Ser-Cys]-Lys), [^{125}I]LTT-SRIF-28 ([Leu^{8},D-Trp^{22},^{125}I-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-D-Trp-Lys-Thr-(^{125}I-Tyr)-Thr-Ser-Cys]-OH). Abbreviations: Asu = amino suberic acid; Aha = amino heptanoic acid; Mpr = 3-mercaptopropionic acid; D-Nal = Naphthyl-D-Ala; Bzl = Benzylsubstituent. GppNHp was from Sigma (St. Louis, MO). BIM 23014, cycloantagonist SA, LTT-SRIF-28, SRIF-14, SRIF-25, and SRIF-28 were purchased from Bachem (Bubendorf, Switzerland), RC160 was purchased from Peninsula Laboratories (Heidelberg, Germany), cortistatin-17 was kindly provided

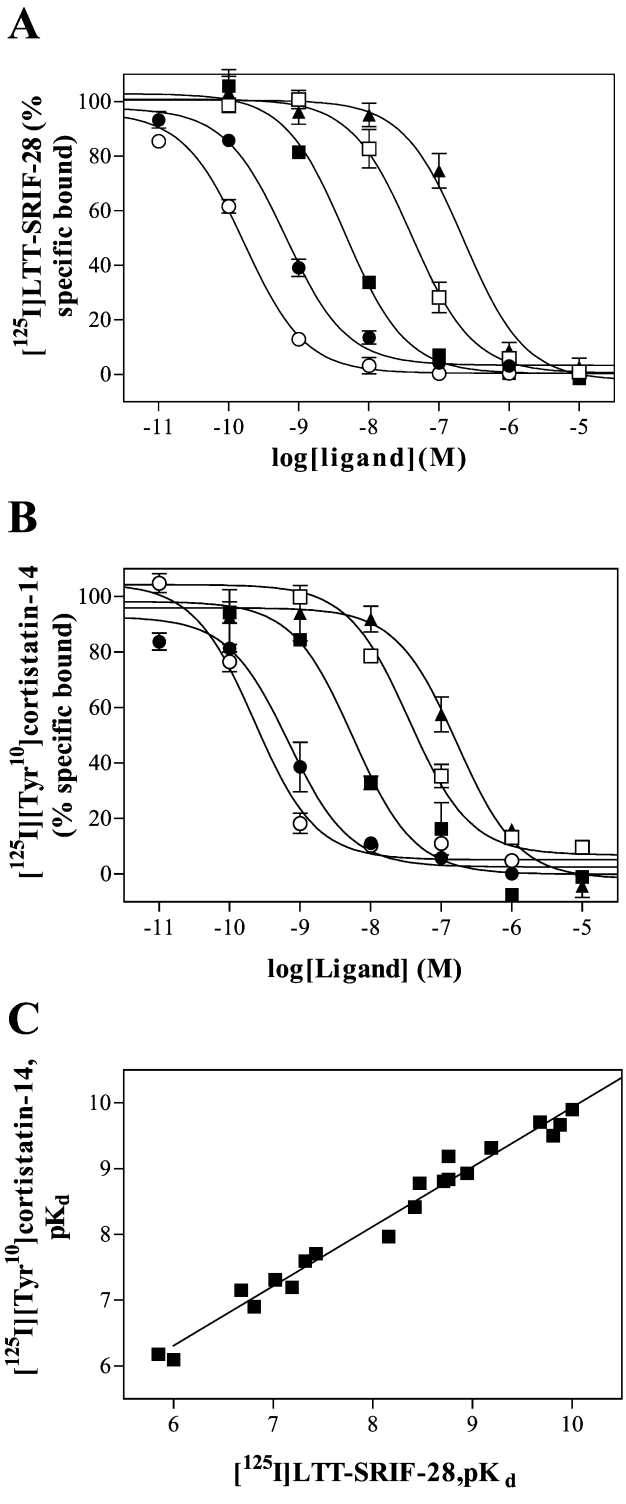

Fig. 3. Examples of competition binding experiments with (A) [^{125}I]LTT-SRIF-28 and (B) [^{125}I][Tyr^{10}]cortistatin-14 to membranes prepared from CCL39-SRE-Luci cells stably expressing goldfish sst$_5$ receptors. Crude membrane preparations were incubated for 1 h at 22 °C with triplicates of radioligand (25–35 pM) in the presence of the indicated concentrations of SRIF-14 (●), LTT-SRIF-28 (○), CGP 23996 (□), cortistatin-14 (■), and BIM 23056 (▲). Data is expressed as percentage specific binding (total and non-specific binding determined in the absence and presence of 10 μM SRIF-14). The plots represent one example of at least three experiments. (C) Correlation analysis of affinity profiles defined by [^{125}I]LTT-SRIF-28 and [^{125}I][Tyr^{10}]cortistatin-14 binding at goldfish sst$_5$ receptors expressed in CCL39-SRE-Luci cells. Data is from Table 1 and compares pK_d values (− log mol l^{-1}) obtained in competition radioligand binding with SRIF ligands.

by Drs. JG. Sutcliffe and L. de Lecea (The Scripps Research Institute, La Jolla, CA) or synthesised at ANAWA (Wangen, Switzerland); cortistatin-14 and [Tyr^{10}]cortistatin-14 were from ANAWA. [Pro^{2}]SRIF-14 and goldfish SRIF-28 (Lin et al., 1999a) were kindly provided by Dr J. Rivier (The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute for Biological Sciences, La Jolla, CA). Other ligands were synthesised at Novartis Pharma (Basel, Switzerland). [^{125}I]LTT-SRIF-28 and [^{125}I][Tyr^{10}]cortistatin-14 were custom synthesised from ANAWA.

3. Results

3.1. Radioligand binding

[^{125}I]LTT-SRIF-28 and [^{125}I][Tyr^{10}]cortistatin-14 labelled gfsst$_5$ binding sites in CCL39-SRE-Luci cells with high affinity and in a saturable manner ([^{125}I]LTT-SRIF-28: $B_{max} = 303 \pm 15$ fmol mg^{-1}, p$K_d = 9.99 \pm 0.19$; [^{125}I][Tyr^{10}]cortistatin-14: $B_{max} = 348 \pm 23$ fmol mg^{-1}, p$K_d = 9.71 \pm 0.08$) (Fig. 1). There was no significant difference between the sites labelled by the two radioligands (Data analysed using independent *t*-tests, *P*: 0.227 and 0.150; $n = 4$ for pK_d and B_{max}, respectively). Non-specific binding was relatively low for both ligands. All saturation curves were monophasic, suggesting labelling of a single population of receptor sites. No specific binding was detected for either radioligand in non-transfected cells.

In the presence of the non-hydrolysable GTP analogue GppNHp (10 μM), both [^{125}I]LTT-SRIF-28 and [^{125}I][Tyr^{10}]cortistatin-14 labelled a smaller population of binding sites (B_{max}: 158 ± 11 and 194 ± 20 fmol mg^{-1}, respectively) (Fig. 2A and B). This represented a significant decrease ($P < 0.002$; $n = 4$) and was approximately two-fold. The affinity of the radioligands was unchanged (*P*: 0.38–0.51; $n = 4$). When increasing concentrations of GppNHp were incubated in the presence of a single concentration of radioligand (Fig. 2C), the binding inhibition was concentration dependent (pEC$_{50}$: 6.39 ± 0.25 and 6.76 ± 0.11 with [^{125}I]LTT-SRIF-28 and [^{125}I][Tyr^{10}]cortistatin-14, respectively). In these experiments, the inhibition produced was significantly greater at [^{125}I][Tyr^{10}]cortistatin-14 than at [^{125}I]LTT-SRIF-28 labelled sites (maximal inhibition E_{max}: 57.9 ± 4.5 and 38.5 ± 3.0%, respectively; $P = 0.025$; $n = 3$).

The pharmacological profiles of [^{125}I]LTT-SRIF-28 and [^{125}I][Tyr^{10}]cortistatin-14 binding at gfsst$_5$ receptors were investigated using SRIF, cortistatin and a selection of their natural and synthetic analogues (Table 1, Fig. 3A and B). The endogenous peptides and their analogues all bound with high affinity (pK_d: 10.0–8.42). The synthetic analogues tended to bind with lower affinity (pK_d: 8.84–5.85). Profiles defined with [^{125}I]LTT-SRIF-28 and [^{125}I][Tyr^{10}]-cortistatin-14 had a highly significant correlation (r^2: 0.98; $P < 0.0001$; $n = 20$) (Fig. 3C) and displayed the following rank order: LTT-SRIF-28>SRIF-28 = gfSRIF-28 ≥ SRIF-25>SRIF-14>[Pro^{2}]SRIF-14 = cortistatin-17 = RC 160 = BIM 23052= [Tyr^{10}]cortistatin-14>cortistatin-14>BIM 23014 ≥ [Tyr^{3}]octreotide = CGP 23996 = octreotide = seglitide>BIM 23056 = L362,855>Cycloantagonist SA = BIM 23030.

To examine species differences in the pharmacological profile of the sst$_5$ receptor, the profile of gfsst$_5$ was compared with those of human (h)sst$_5$ (Siehler et al., 1999a) and mouse (m)sst$_5$ receptors (Feuerbach et al., 2000) defined using [^{125}I]LTT-SRIF-28 and [^{125}I][Tyr^{10}]cortistatin-14 in CCL39 cells (Table 2). Large species differences were seen between the profiles of gfsst$_5$ and those of msst$_5$ and hsst$_5$

Table 2
Comparison of goldfish, mouse and human sst$_5$ receptor binding profiles using [^{125}I]LTT-SRIF-28 and [^{125}I][Tyr^{10}]cortistatin-14

	[^{125}I]LTT-SRIF-28			[^{125}I][Tyr^{10}]cortistatin-14		
	Goldfish	Human	Mouse	Goldfish	Human	Mouse
LTT-SRIF-28	10.00 ± 0.15	8.47 ± 0.02		9.90 ± 0.08	8.12 ± 0.01	
SRIF-28	9.88 ± 0.19	9.39 ± 0.22	9.75 ± 0.16	9.67 ± .010	9.18 ± 0.19	9.94 ± 0.05
SRIF-14	9.19 ± 0.05	9.53 ± 0.13	7.73 ± 0.04	9.32 ± 0.19	9.01 ± 0.24	9.02 ± 0.02
Cortistatin-17	8.76 ± 0.10	9.54 ± 0.10	8.85 ± 0.03	9.19 ± 0.03	9.37 ± 0.09	9.34 ± 0.04
RC 160	8.76 ± 0.47	7.51 ± 0.06	8.38 ± 0.14	8.84 ± 0.32	7.27 ± 0.11	9.14 ± 0.02
BIM 23052	8.71 ± 0.10	7.92 ± 0.19	8.90 ± 0.13	8.81 ± 0.11	7.45 ± 0.24	9.68 ± 0.05
[Tyr^{10}]Cortistatin-14	8.47 ± 0.15	8.67 ± 0.24	8.34 ± 0.09	8.78 ± 0.11	8.06 ± 0.40	8.89 ± 0.04
Cortistatin-14	8.42 ± 0.13	8.71 ± 0.02	8.68 ± 0.06	8.42 ± 0.15	8.40 ± 0.04	9.07 ± 0.08
BIM 23014	8.16 ± 0.08	7.76 ± 0.13	8.10 ± 0.14	7.97 ± 0.35	7.38 ± 0.19	8.92 ± 0.07
[Tyr^{3}]Octreotide	7.43 ± 0.10	6.49 ± 0.01		7.71 ± 0.09	6.00 ± 0.07	
CGP 23996	7.32 ± 0.11	6.59 ± 0.41	7.96 ± 0.07	7.60 ± 0.10	6.67 ± 0.24	8.47 ± 0.01
Octreotide	7.19 ± 0.12	7.17 ± 0.30	7.70 ± 0.05	7.20 ± 0.17	7.31 ± 0.18	9.04 ± 0.05
Seglitide	7.02 ± 0.13	8.70 ± 0.26	6.70 ± 0.01	7.31 ± 0.02	9.14 ± 0.30	7.90 ± 0.05
BIM 23056	6.81 ± 0.08	7.17 ± 0.05	7.50 ± 0.13	6.90 ± 0.08	6.68 ± 0.05	7.82 ± 0.06
L362,855	6.68 ± 0.13	7.17 ± 0.30	7.70 ± 0.14	7.15 ± 0.10	7.17 ± 0.12	8.45 ± 0.02
Cycloantagonist SA	6.00 ± 0.07	6.38 ± 0.23	6.03 ± 0.15	6.10 ± 0.05	6.02 ± 0.11	6.79 ± 0.04
BIM 23030	5.85 ± 0.14	6.02 ± 0.09	7.08 ± 0.13	6.18 ± 0.05	5.56 ± 0.17	7.62 ± 0.04

Human data is from Siehler et al. (1999a) and mouse data is from Feuerbach et al. (2000), all receptors are expressed in CCL39 cells. The data are expressed as pK_d values ($-\log$ mol l^{-1}) ± S.E.M. of at least three determinations.

receptors with both radioligands. This was more pronounced when comparing $hsst_5$ and $gfsst_5$ profiles (r^2: 0.52–0.60; $P<0.001$; $n=17$), $hsst_5$ pK_d values appeared to be generally lower than, or equal to, those of $gfsst_5$ with the exception of seglitide. There was a better correlation between $gfsst_5$ and $msst_5$ receptor profiles (r^2: 0.72–0.78; $P<0.0001$; $n=15$), with the synthetic ligands tending to have slightly higher affinity for $msst_5$ than $gfsst_5$ receptors.

3.2. Functional assays

The G-protein binding potential of the goldfish sst_5 receptor was further investigated by measuring agonist-stimulated binding of the non-hydrolysable GTP analogue [^{35}S]GTPγS. In $gfsst_5$ transfected CCL39 cells, SRIF-14 produced a two- to three-fold increase in [^{35}S]GTPγS binding over basal levels whereas no increase was seen in non-transfected CCL39-SRE-Luci cells. In non-transfected CCL39-SRE-Luci cells, basal [^{35}S]GTPγS binding was 20% lower than that of CCL39-SRE-Luci-$gfsst_5$ (data not shown). Stimulation of [^{35}S]GTPγS binding was completely abolished (E_{max}: 0.22 ± 9.0 relative intrinsic activity) in cells which were pre-incubated with pertussis toxin (100 ng ml^{-1}) for 18 h (Fig. 4A). The effects of NaCl concentration on [^{35}S]GTPγS binding was investigated by measuring basal [^{35}S]GTPγS binding in the presence of increasing NaCl concentrations (Fig. 4B). Specific bound [^{35}S]GTPγS decreased as NaCl concentrations increased.

SRIF, cortistatin and their natural and synthetic analogues produced a concentration-dependent increase in [^{35}S]GTPγS binding (Table 3, Fig. 5A). The profile was similar to that achieved in radioligand binding studies (r^2: 0.82; $P<0.0001$; $n=20$), although relative potency (pEC_{50}) was reduced by a factor of 1–2 log units. With respect to relative efficacy, natural SRIF ligands acted as full agonists in relation to SRIF-14 (E_{max}: 86–114% relative intrinsic activity) with the exception of cortistatin-14 which showed partial agonism (E_{max}: 69.8% relative intrinsic activity). Of the synthetic analogues, RC 160, BIM 23052, BIM 23014, [Tyr^3]octreotide and L362,855 acted as full agonists (E_{max}: 94.8 ± 1.0%, 114.0 ± 12.7%, 96.8 ± 9.9%, 92.5 ± 5.7%, 105.2 ± 16.0% relative intrinsic activity, respectively), the remaining analogues displaying partial agonism (E_{max}: 31–82% relative intrinsic activity). In contrast, BIM 23056 showed inverse agonism (E_{max}: −43.9 ± 6.5% relative intrinsic activity), and antagonised the effects of SRIF-14 in [^{35}S]GTPγS binding with a pK_B of 6.74 ± 0.23 (Fig. 5B).

The CCL39-SRE-Luci cell line used to express $gfsst_5$ has been engineered to express the luciferase reporter gene

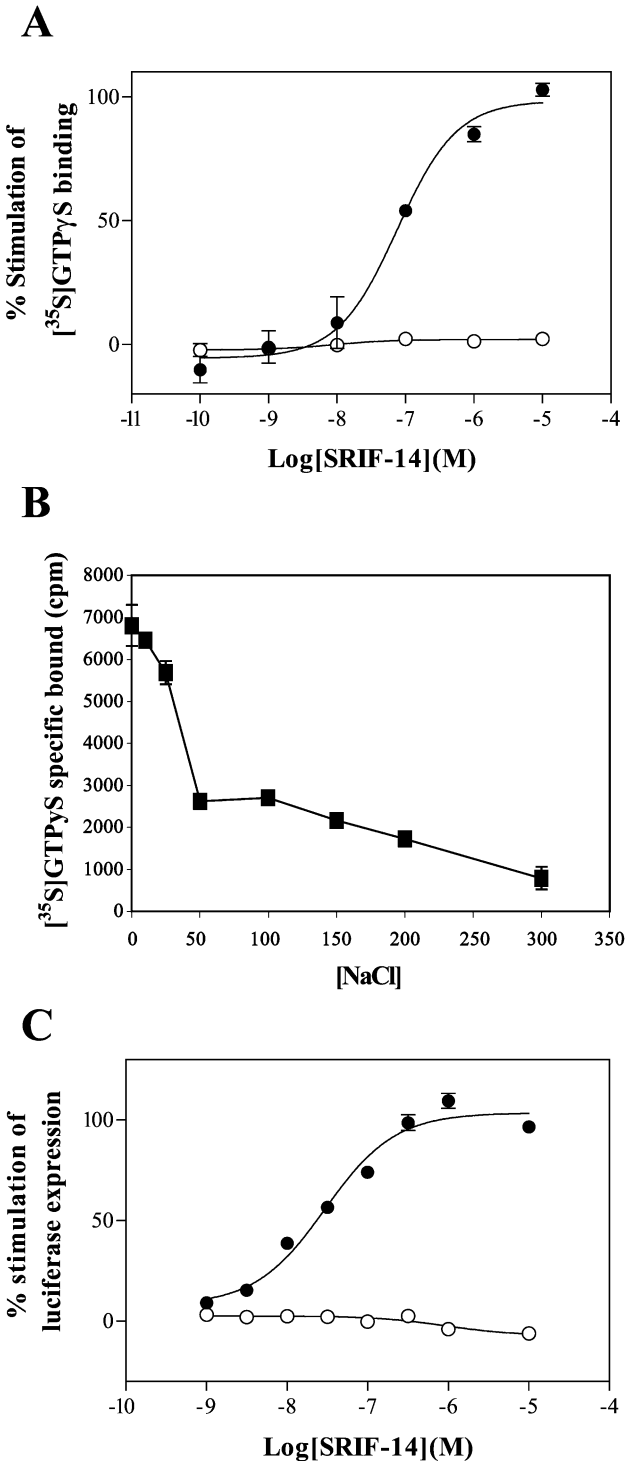

Fig. 4. The effects of pertussis toxin on SRIF-14-stimulated [^{35}S]GTPγS binding and luciferase reporter gene activity in CCL39-SRE-Luci cells stably expressing goldfish sst_5 receptors. Cells were incubated with (○) or without (●) pertussis toxin (100 ng ml^{-1}) for 18 h before harvesting. (A) Microsome preparations were incubated for 30 min at 22 °C with triplicates of [^{35}S]GTPγS and the indicated concentrations of SRIF-14. Data is expressed as percentage of specific [^{35}S]GTPγS binding stimulated by 10 μM SRIF-14, which was included in each experiment. (C) Cells were incubated at 37 °C, 5% CO_2, 95% relative humidity in triplicate at the indicated concentrations of SRIF-14. Cells were lysed after 5 h and luciferase activity was determined. Data is expressed as % stimulation of maximal SRIF-14 effect. (B) Effect of sodium chloride concentration on basal [^{35}S]GTPγS binding in CCL39-SRE-luci cells stably expressing goldfish sst_5 receptors. Microsome preparations were incubated for 30 min at 22 °C with triplicates of [^{35}S]GTPγS using the NaCl concentration as indicated. Data represent the percentage of [^{35}S]GTPγS specific bound. All plots represent one example of three experiments.

Table 3
Stimulation of [^{35}S]GTPγS binding by SRIF ligands acting at goldfish sst$_5$ receptors expressed in CCL39-SRE-Luci cells

	PEC_{50}	E_{max}
LTT-SRIF-28	8.54 ± 0.09	113.9 ± 3.6
SRIF-28	8.09 ± 0.10	100.3 ± 2.5
GF SRIF-28	8.26 ± 0.13	113.9 ± 10.5
SRIF-25	8.23 ± 0.12	106.2 ± 5.9
SRIF-14	7.20 ± 0.07	99.5 ± 1.2
GF SRIF-14	7.27 ± 0.11	93.2 ± 2.8
Cortistatin-17	7.74 ± 0.67	86.4 ± 2.6
RC 160	6.81 ± 0.17	94.8 ± 1.0
BIM 23052	6.91 ± 0.01	114.0 ± 12.7
[Tyr^{10}]Cortistatin-14	6.80 ± 0.09	92.2 ± 3.3
Cortistatin-14	6.95 ± 0.14	69.8 ± 9.3
BIM 23014	6.71 ± 0.15	96.8 ± 9.9
[Tyr^{3}]Octreotide	6.15 ± 0.10	92.5 ± 5.7
CGP 23996	6.18 ± 0.12	82.3 ± 4.9
Octreotide	5.92 ± 0.09	70.5 ± 5.6
Seglitide	5.69 ± 0.07	70.4 ± 4.3
BIM 23056	6.03 ± 0.23	− 43.9 ± 6.5
L362,855	5.16 ± 0.11	105.2 ± 16.0
Cycloantagonist SA	6.44 ± 0.75	30.6 ± 2.8
BIM 23030		8.5 ± 2.1

The data are expressed as pEC_{50} values (− log of the molar concentration required for a half-maximal effect) and E_{max} (% stimulation of the maximal effect produced by SRIF-14). Data are listed as means ± S.E.M. of at least four determinations.

under the control of the serum responsive element (Table 4). This was used as a measure of functional activity of the receptor. The clone that was used for pharmacological characterisation produced a five-fold increase in luciferase expression over basal when stimulated with SRIF-14, whereas no increase was seen in non-transfected CCL39-SRE-Luci cells (data not shown). Pertussis toxin pre-incubation (18 h, 100 ng ml^{-1}) abolished the ability of SRIF-14 to stimulate luciferase expression (E_{max}: − 4.5 ± 2.4% relative intrinsic activity) (Fig. 4C).

The effects of SRIF, cortistatin and their natural and synthetic analogues were similar to those in [^{35}S]GTPγS binding (Fig. 5C). Concentration-dependent stimulation of luciferase expression was seen which displayed similar potency profiles to those of [^{35}S]GTPγS ($r^2 = 0.90$; $P < 0.0001$; $n = 20$) and radioligand binding ($r^2 = 0.94$; $P < 0.0001$; $n = 20$), with relative potency being 1–2 log units lower than radioligand binding. Relative efficacy profiles of the ligands did not correlate well with those of [^{35}S]GTPγS binding ($r^2 = 0.49$; $P < 0.0001$; $n = 20$). As with [^{35}S]GTPγS binding, natural ligands acted as full agonists in relation to SRIF-14 (E_{max}: 96–117% relative intrinsic activity) with the exception of cortistatin-14 (E_{max}: 66.8% relative intrinsic activity). Synthetic analogues, however, showed only partial agonism (E_{max}: 20–65% relative intrin-

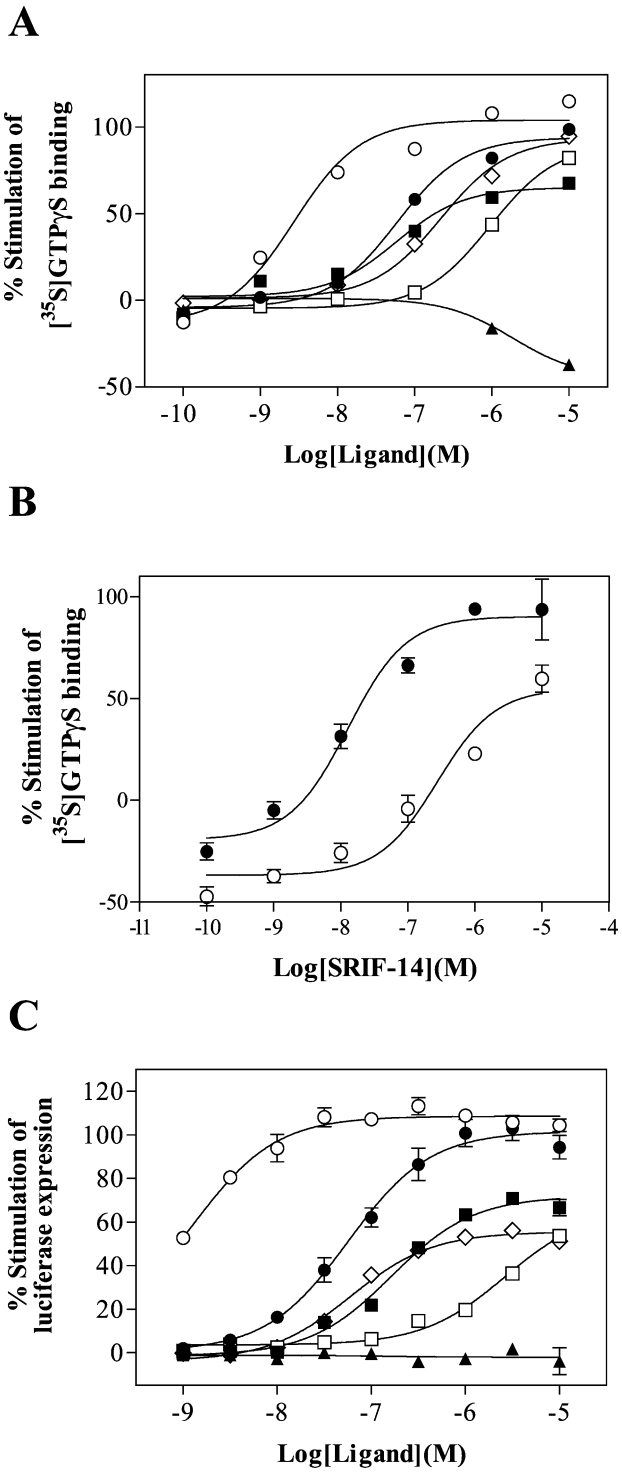

Fig. 5. Examples of agonist-stimulated [^{35}S]GTPγS binding and luciferase reporter gene activity in CCL39-SRE-Luci cells stably expressing goldfish sst$_5$ receptors. Microsome preparations were incubated for 30 min at 22 °C with triplicates of [^{35}S]GTPγS and the indicated concentrations of (A) LTT-SRIF-28 (○), SRIF-14 (●), cortistatin-14 (■), CGP 23996 (□), BIM 23056 (▲) and RC 160 (◇). (B) SRIF-14, without (●) or with (○) BIM 23056 (10 μM). Data is expressed as percentage of specific [^{35}S]GTPγS binding stimulated by 10 μM SRIF-14, which was included in each experiment. (C) Cells were incubated at 37 °C, 5% CO_2, 95% relative humidity with triplicates of the indicated concentrations of LTT-SRIF-28 (○), SRIF-14 (●), cortistatin-14 (■), CGP 23996 (□), BIM 23056 (▲) and RC 160 (◇). Cells were lysed after 5 h and luciferase activity was determined. Data is expressed as % stimulation of maximal SRIF-14 effect. All plots represent one example of at least three experiments.

Table 4
Stimulation of luciferase reporter gene activity by SRIF ligands acting at goldfish sst_5 receptors expressed in CCL39-SRE-Luci cells

	pEC_{50}	E_{max}
LTT-SRIF-28	8.93 ± 0.10	117.4 ± 5.9
SRIF-28	8.37 ± 0.13	112.3 ± 5.1
GF SRIF-28	8.48 ± 0.16	102.8 ± 4.8
SRIF-25	8.43 ± 0.16	104.7 ± 4.8
SRIF-14	7.39 ± 0.08	100
GF SRIF-14	6.99 ± 0.04	96.4 ± 4.4
Cortistatin-17	7.04 ± 0.23	99.2 ± 5.4
RC 160	7.20 ± 0.16	49.8 ± 4.3
BIM 23052	6.90 ± 0.14	65.8 ± 4.4
[Tyr^{10}]Cortistatin-14	6.53 ± 0.07	81.8 ± 4.8
Cortistatin-14	6.70 ± 0.17	66.8 ± 2.3
BIM 23014	6.54 ± 0.30	35.8 ± 4.7
[Tyr^{3}]Octreotide	5.64 ± 0.23	54.0 ± 1.5
CGP 23996	5.59 ± 0.11	65.3 ± 1.5
Octreotide	5.64 ± 0.08	34.0 ± 2.5
Seglitide	5.28 ± 0.09	45.0 ± 4.4
BIM 23056		5.9 ± 0.8
L362,855	5.69 ± 0.15	20.8 ± 2.6
Cycloantagonist SA		2.05 ± 3.4
BIM 23030		2.18 ± 1.45

The data are expressed as pEC_{50} values (− log of the molar concentration required for a half-maximal effect) and E_{max} (% stimulation of maximal effect produced by SRIF-14). Data are listed as means ± S.E.M. of at least four determinations.

sic activity), with a few (Cycloantagonist SA, BIM 23030, BIM 23056) having no effect.

4. Discussion

The somatostatin neuropeptide family exerts its effects by binding to G protein-coupled receptors of which five subtypes have been cloned from several mammalian species and sst_{1A}, sst_{1B}, sst_2, sst_3 and sst_5 receptors have been cloned from teleost fish species (Bell and Reisine, 1993; Hoyer et al., 1994, 1995; Siehler et al., 1999b; Lin et al., 2002, in press). In this study, a type 5 somatostatin receptor isolated from goldfish has been expressed and pharmacologically characterised in CCL39-SRE-Luci cells.

The agonist radioligands [^{125}I]LTT-SRIF-28 and [^{125}I][Tyr^{10}]cortistatin-14 were used in saturation experiments to determine levels of receptor expression. Both ligands bind to a homogenous population of receptors with nM K_ds, which can be considered to be high affinity. A similar density of receptors is labelled by both ligands with expression levels in the range 300−350 fmol mg^{-1}. These levels are similar to those of other SRIF receptors expressed in CCL39 cells, and to levels of receptors labelled in peripheral tissues and brain membranes, suggesting that they can be considered close to physiological (Siehler et al., 1999a,b; Feuerbach et al., 2000).

Saturation experiments were repeated in the presence of the non-hydrolysable GTP analogue GppNHp in order to examine the G-protein coupling of the receptor population being labelled. GppNHp produces a decrease in the apparent receptor density labelled by both radioligands which is about two-fold. No change in the affinity of the ligands for the receptor is seen. In a separate set of experiments where increasing concentrations of GppNHp were incubated with a single concentration of radioligand, partial inhibition of binding is seen. Assuming that the concentration of GppNHp used was high enough to saturate the G proteins, as suggested by the sigmoidal inhibition curves, this suggests that the agonist radioligands used are labelling a population of both G-protein-coupled, and G-protein-uncoupled receptors, or at least receptors in different states that may or may not be coupled to G proteins. This has been shown to occur with radioligand binding at other SRIF receptors, including sst_5 receptors from human, rat and mouse, and some fish receptors (O'Carroll et al., 1992; Panetta et al., 1994; Siehler et al., 1999a,b; Feuerbach et al., 2000).

To characterise the pharmacological features of the $gfsst_5$ receptor, competition binding studies were carried out using the radioligands [^{125}I]LTT-SRIF-28 and [^{125}I][Tyr^{10}]cortistatin-14, and a number of natural and synthetic SRIF ligands. The rank orders of affinity defined by both radioligands are almost identical. SRIF, cortistatin and their natural analogues all bind to $gfsst_5$ with nM affinity. SRIF-28 and LTT-SRIF-28 bind with four- to five-fold higher affinity than SRIF-14 which is consistent with this receptor being an sst_5 receptor: sst_5 receptors in human, mouse and rat have been shown to prefer SRIF-28 to SRIF-14, whereas no distinction between the two is seen at sst_{1-4} (Meyerhof et al., 1992; O'Carroll et al., 1992, 1994; Yamada et al., 1993; Panetta et al., 1994; Patel and Srikant, 1994; Patel et al., 1994; Raulf et al., 1994; Baumeister et al., 1998). The two goldfish peptides [Pro^2]SRIF-14 and goldfish SRIF-28, have affinities similar to their mammalian homologues. The synthetic peptides tested here tend to bind with lower affinities than SRIF-14, with the exception of BIM 23052, BIM 23014, CGP 23996 and the putative sst_2 receptor selective agonist RC 160.

Comparison of profiles defined with [^{125}I]LTT-SRIF-28 and [^{125}I][Tyr^{10}]cortistatin-14 at $gfsst_5$, with those defined at human and mouse sst_5 in CCL39 cells (Siehler et al., 1999a; Feuerbach et al., 2000) shows marked species differences which are greatest between goldfish and human receptors. Synthetic ligands such as seglitide, L362,855, and octreotide, which have been shown to have high affinity for sst_5 in CCL39 cells and in other cell lines, tend to have relatively low (μM) affinity for the goldfish sst_5 receptor. This may be expected when comparing receptors from mammalian and non-mammalian vertebrates. However, the sst_5 receptor is notorious for large species differences between ligand binding profiles which are not observed as markedly between other SRIF receptor subtypes: for example, $hsst_5$ has a 160-fold greater affinity for octreotide than rat sst_5 (O'Carroll et al., 1994). In fact, the affinity profiles of either mouse or human sst_5 expressed in CCL39 cells correlate better with

the goldfish sst_5 than they do with each other ($r^2 = 0.385$ [^{125}I][Tyr^{10}]cortistatin-14 and 0.323 [^{125}I]LTT-SRIF-28, (Feuerbach et al., 2000)) suggesting that the differences between the fish and mammalian receptors may simply be a fact of SRIF receptor diversity rather than due to a difference between mammalian and non-mammalian receptors.

Agonist-dependent stimulation of [^{35}S]GTPγS binding and serum response element-driven luciferase expression was used to examine the functional effects of SRIF ligands at the $gfsst_5$ receptor. Activation of $gfsst_5$ by SRIF-14 leads to stimulation of [^{35}S]GTPγS binding with a maximal stimulation of about 200% over basal. This was completely blocked by pertussis toxin (100 ng ml^{-1}, 18 h) suggesting the G proteins involved are G_i/G_o proteins (Reisine, 1990).

The relative potencies of SRIF ligands in [^{35}S]GTPγS binding studies are similar to profiles defined in radioligand binding (Fig. 6). Potency values achieved for individual ligands are between 10- and 1000-fold lower than affinity values defined in binding studies, as has been observed previously for [^{35}S]GTPγS binding at somatostatin receptors, and other receptors such as 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D} (Pauwels et al., 1997a,b; Stanton and Beer, 1997; Williams et al., 1997; Siehler and Hoyer, 1999). The relative efficacy of all ligands is expressed as a percentage of the response of SRIF-14, which was assumed to be a full agonist (100% efficacy). SRIF-28 and its analogues (including the goldfish peptide) have a higher efficacy than SRIF-14. The synthetic analogues BIM 23052 and L362,855 also stimulate [^{35}S]GTPγS binding to a greater degree than SRIF-14, although with lower potency. Most synthetic analogues tested are full or nearly full agonists, with maximal stimulation of greater than 70% compared to SRIF-14. Cycloantagonist SA and BIM 23030 are the only exceptions, showing partial agonism and no activity, respectively.

BIM 23056 appears to act as an inverse agonist in the $gfsst_5$ [^{35}S]GTPγS binding assay. It also acts as a potent antagonist against SRIF-14-stimulated [^{35}S]GTPγS binding. BIM 23056 has previously been shown to act as an antagonist at SRIF-14-stimulated [^{35}S]GTPγS binding at $hsst_5$ in CHO-K1 and CCL39 cells (Williams et al., 1997; Siehler and Hoyer, 1999), as well as SRIF-14-stimulated inositol phosphate accumulation and intracellular Ca^{2+} accumulation at $hsst_5$ in CHO-K1 cells (Wilkinson et al., 1996, 1997). However, BIM 23056 alone has previously been found to be devoid of activity in functional assays, including [^{35}S]GTPγS binding, at sst_5 receptors (Wilkinson et al., 1997; Williams et al., 1997; Siehler and Hoyer, 1999), with the exception of $msst_5$ where it was shown to have partial agonist effects on luciferase expression ($E_{max} = 37\%$, (Feuerbach et al., 2000)).

At $gfsst_5$ BIM 23056 appears to be acting as a "inverse antagonist" rather than as a neutral one, as has been described for other ligand/receptor interactions, including methiothepin and spiperone at 5-HT_{1A} receptors (Newman-Tancredi et al., 1997a,b; Stanton and Beer, 1997; McLoughlin and Strange, 2000) and rauwolscine at α_2-adrenoceptors (Tian et al., 1994). This effect of ligands is attributed to their ability to stabilise receptors in an inactive state. It assumes that the receptors have a basal level of activity, even in the absence of agonist, and that the inverse agonist is inhibiting constitutive G protein coupling/activation of the receptors (Costa et al., 1992; Tian et al., 1994; Cosi and Koek, 2000; McLoughlin and Strange, 2000; Sim-Selley et al., 2001).

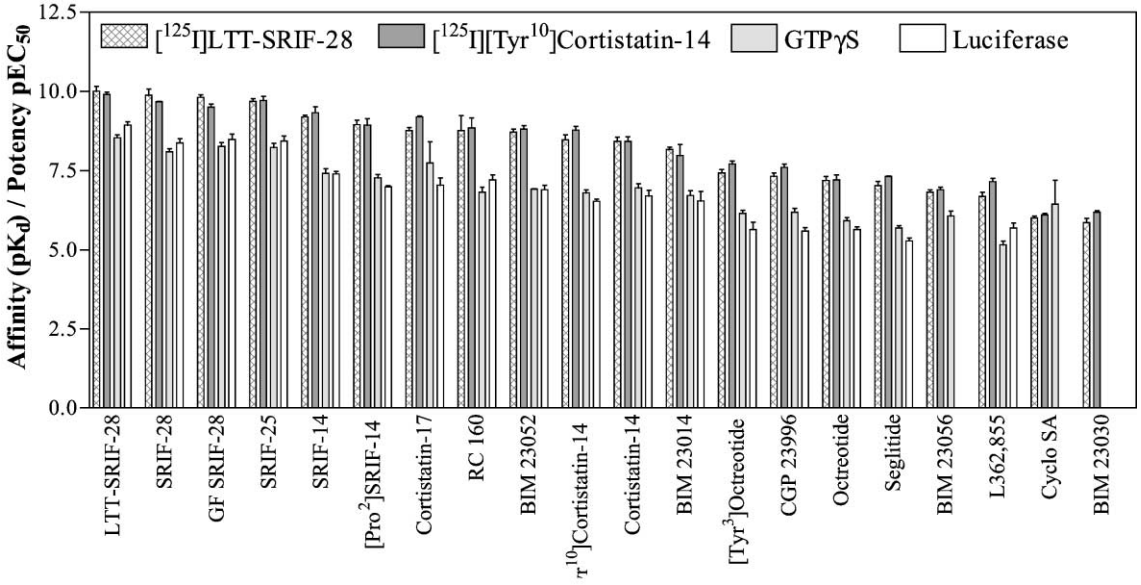

Fig. 6. Comparison of affinity/potency profiles determined in radioligand binding, [^{35}S]GTPγS binding, and luciferase expression assays in CCL39-SRE-Luci cells stably expressing goldfish sst_5 receptors. Data is from Tables 1, 3 and 4 and is expressed as pK_d (radioligand binding), or pEC_{50} ([^{35}S]GTPγS binding, and luciferase expression) and represents the means ± S.E.M. of at least three experiments.

In this study, evidence for the existence of constitutive G-protein coupling/activation of the receptor is shown in two ways. Firstly, basal [^{35}S]GTPγS binding is 20% lower in non-transfected CCL39-SRE-Luci cells than in cells transfected with gfsst$_5$, suggesting that at least a proportion of the receptors are existing in a G-protein-coupled state even in the absence of ligand, as described for 5-HT$_{1A}$ receptors in CHO and HeLa cells (Cosi and Koek, 2000; McLoughlin and Strange, 2000). Secondly, increasing concentrations of NaCl decreased the levels of basal [^{35}S]GTPγS binding in these cells. Na^+ inhibits spontaneous activity of many G_i/G_o coupled receptors (Sim-Selley et al., 2001), probably by acting as a negative modulator of receptor/G-protein pre-coupling equilibrium in the ternary complex model (Horstman et al., 1990; Costa et al., 1992; Raynor et al., 1993a,b; Cosi and Koek, 2000). Therefore, the sensitivity of basal [^{35}S]GTPγS to Na^+ in gfsst$_5$ transfected cells may be due to Na^+ inducing uncoupling of receptor/G-protein, suggesting that these receptors are indeed pre-coupled to G proteins.

The CCL39 cell line used to express the receptor was previously transfected with plasmids containing the serum response element upstream of the luciferase reporter gene. The gfsst$_5$ receptor stimulates luciferase gene expression by a five-fold increase over basal levels, as has previously been shown with the msst$_5$ (Feuerbach et al., 2000). This was completely blocked by pre-incubation of pertussis toxin (100 ng ml^{-1}, 18 h), suggesting it occurs via G_i/G_o proteins. The relative potency profile of SRIF ligands in stimulating luciferase expression at gfsst$_5$ correlates highly to affinity profiles defined in radioligand binding and the potency profile in [^{35}S]GTPγS binding (Fig. 6), with absolute potencies being 10–1000 fold lower than binding affinities as previously discussed for [^{35}S]GTPγS binding.

As with [^{35}S]GTPγS binding, the relative efficacy of all ligands is expressed as a percentage of the response of SRIF-14. SRIF-28 and its analogues are more efficacious than SRIF-14 in stimulating luciferase expression. In general however, synthetic analogues show only partial agonism in stimulating luciferase expression, with maximal stimulation of synthetic analogues reaching only 65% of that of SRIF-14; this was achieved by BIM 23052 and CGP 23996. BIM 23056, which displays inverse agonism at gfsst$_5$ receptors expressed in CCL39-SRE-Luci cells in [^{35}S]GTPγS binding, appears to have negligible activity in stimulating luciferase expression. The reason for this is unclear, at 5-HT$_{1B/1D}$ receptors methiothepin and ketanserin have been shown to act as inverse agonists when measuring [^{35}S]GTPγS binding, which had not been previously observed in cAMP and mitogenic assays (Pauwels et al., 1997a), suggesting that [^{35}S]GTPγS binding may be a more sensitive assay to differentiate between functional responses of ligands. It is also possible that precoupling of receptors does not occur, or occurs to a lower level in intact cells (Costa et al., 1992). Should this be the case, the inverse agonism of BIM 23056 in this system may have no physiological relevance and BIM 23056 may be considered purely as an antagonist in vivo.

The relative efficacy profiles from the two functional assays are highly divergent, (see Fig. 7). Although all ligands have a higher E_{max} when stimulating [^{35}S]GTPγS

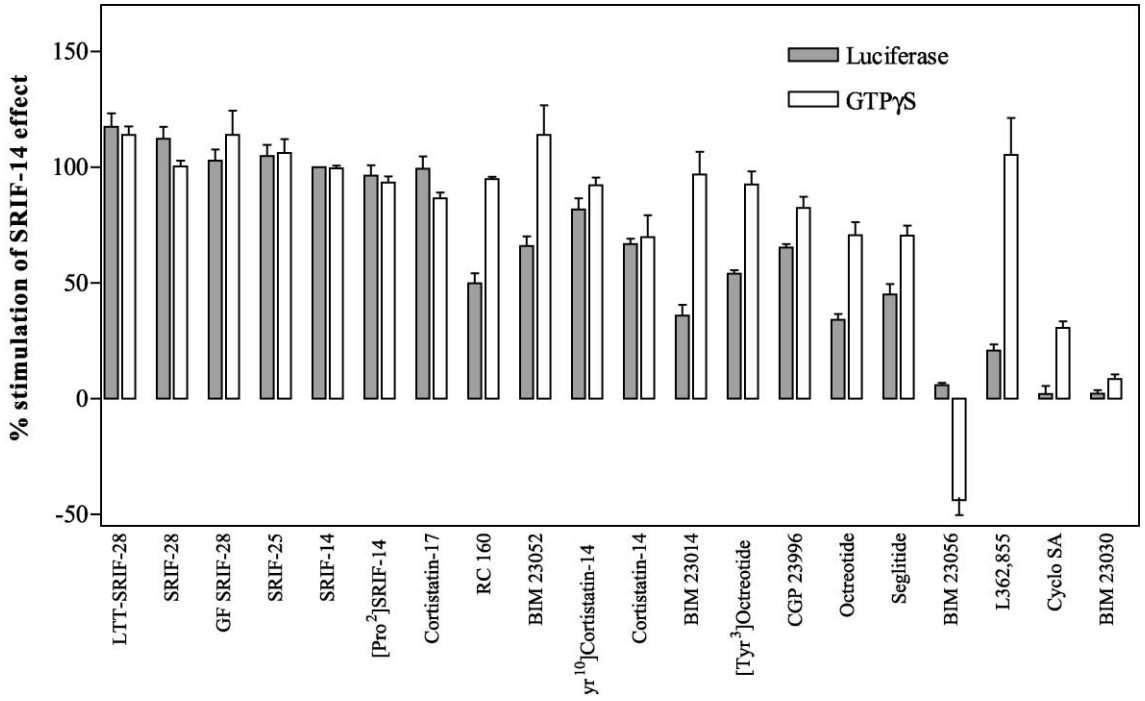

Fig. 7. Comparison of agonist-stimulated [^{35}S]GTPγS binding and luciferase expression CCL39-SRE-Luci cells stably expressing goldfish sst$_5$ receptors. Data is expressed as E_{max} (% stimulation of SRIF-14 effect) and represents the means ± S.E.M. of at least three experiments.

binding, the ratio between the E_{max} of [^{35}S]GTPγS binding, and that of luciferase expression, is not consistent for all ligands. Some have similarly high ranking in both assays, whereas others have very different effects in the two assays, for example L362,855 has an E_{max} of 105% in [^{35}S]GTPγS, and 21% in luciferase, thus being relatively inactive in stimulating luciferase expression.

[^{35}S]GTPγS binding may be expected to provide different estimates of efficacy compared to other assays as a result of different degrees of amplification (McLoughlin and Strange, 2000). This cannot, however, account for the divergent profiles of ligands between [^{35}S]GTPγS assays and luciferase expression. It appears that some agonists are showing selectivity for one transduction pathway over another. Luciferase expression has been shown to occur through the activation of mitogen activated protein kinase (MAP kinase) (Lowe et al., 1997; Wang et al., 1997; Stepan et al., 1999), which, in G_i-coupled receptors, is often (although not always) mediated via Gβ1γ subunits (Crespo et al., 1994; Clapham and Neer, 1997; Gutkind, 1998; Murga et al., 1999). GTPγS, in contrast, binds to the Gα subunit of the G protein. It is possible that the effects observed at the $gfsst_5$ receptor are due to the ability of the ligands to differentially activate the receptor, having different efficacy at activating some pathways (e.g. Gβ1γ and MAP kinase) than others (e.g. Gα). Similar findings have been reported with other G-protein-coupled receptors, particularly for 5-HT_{2A} and 5-HT_{2C} (Berg et al., 1998, 1999), where it has been suggested that phospholipase C stimulation and Ca^{2+} mobilisation go in parallel (as expected, since Ca^{2+} mobilisation is triggered by inositol triphosphate accumulation), whereas phospholipase A_2 activity showed a different rank order of relative efficacy; and in $fsst_3$ and $hsst_5$ where different agonist radioligands labelled apparently different populations of receptors (Siehler et al., 1998, 1999a,b). These results do not support the allosteric ternary complex model (De Lean et al., 1980; Samama et al., 1993; Lefkowitz et al., 1993) but suggest (at least) a three-state model or even a multiple-state model in which the agonist-receptor complex may adopt different conformations depending on agonist and G-protein (Scaramellini and Leff, 1998).

In conclusion, the two functional assays tested here suggest that the receptors are in a relatively low affinity state compared to binding studies; this is similar to what has been reported at 5-HT_{1A} receptors where [^{3}H]8-OH-DPAT binding identifies sites which are in a higher affinity "state" than can be estimated using adenylate cyclase inhibition (Schoeffter et al., 1997). The apparent potency estimated in the functional tests is less than affinity values obtained in agonist binding studies. What may be surprising is that although luciferase expression may be less prone to receptor reserve (more peptides act as partial agonists), than the [^{35}S]GTPγS binding assay (most peptides are close to full agonists), the apparent potency estimated when measuring luciferase expression is higher than that found when measuring [^{35}S]GTPγS binding. L362,855, Cycloantagonist SA, and BIM 23056 show almost no activity when stimulating luciferase expression, whereas they display significant agonism in stimulating [^{35}S]GTPγS binding, with the exception of BIM 23056 which is an inverse agonist on [^{35}S]GTPγS binding. The rank order of relative efficacy is not consistent between the two assays, and does not fit classical receptor theory. Indeed, if classical theory was at play, one would expect that as receptor reserve becomes less pronounced, there would be a decrease in apparent potency, and that when there is no receptor reserve at all, a decrease in relative efficacy. This is clearly not what is observed here, and the behaviour of the various compounds tested does not fit into a general pattern, rather it seems to be ligand-dependent, i.e. each compound acting in its own right. Considering the three-state model, it could be rationalised that a compound may prefer one pathway over another, with another compound preferring the other pathway, or showing no preference. Although this model may apply, there is one limitation, in that there is no evidence for depletion; i.e. a ligand that drives or favours one active state over the other may act as a full agonist at the first but not at the second state: this is not the case here, since the endogenous agonists act as full agonists in both tests, except for cortistatin-14. We may conclude from this that there are presumably numerous conformational states of the goldfish sst_5 receptors, and that there may be several states that allow full agonism at different pathways, assuming that what is being measured here is directly linked to the formation of the agonist-receptor-G protein complex. This would explain why SRIF-14 or SRIF-28 are generally full agonists at the SRIF receptors whichever transduction pathway is being studies, whereas other compounds (e.g. BIM 23056) may show at the same receptor inverse agonism at one pathway, neutral antagonism at a second and partial or even full agonism at a third pathway, all driven by the same receptor in the same cell.

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