British Journal of Pharmacology (2003) 139, 109–121
www.nature.com/bjp

Pharmacological characterisation of native somatostatin receptors in AtT-20 mouse tumour corticotrophs

[1,2]Davide Cervia, [2]Caroline Nunn, [2]Dominique Fehlmann, [2]Daniel Langenegger, [2]Edi Schuepbach & *,[2]Daniel Hoyer

[1]Dipartimento di Fisiologia e Biochimica 'G. Moruzzi', Università di Pisa, 56127 Pisa, Italy and [2]Nervous System Research, Novartis Pharma AG, CH-4002 Basel, Switzerland

1 The mouse corticotroph tumour cell line AtT-20 is a useful model to investigate the physiological role of native somatostatin (SRIF, Somatotropin release inhibitory factor) receptor subtypes (sst_1 – sst_5). The objective of this study was to characterise the pharmacological features and the functional effects of SRIF receptors expressed by AtT-20 cells using radioligand binding and cAMP accumulation.

2 [^{125}I]LTT-SRIF-28, [^{125}I]CGP 23996, [^{125}I]Tyr^{10}-cortistatin-14 and [^{125}I]Tyr^3-octreotide labelled SRIF receptor binding sites with high affinity and in a saturable manner ($B_{max} = 315$, 274, 239 and 206 fmol mg^{-1}, respectively). [^{125}I]LTT-SRIF-28 labels significantly more sites than [^{125}I]Tyr^{10} -cortistatin-14 and [^{125}I]Tyr^3 -octreotide as seen previously in cells expressing pure populations of sst_2 or sst_5 receptors.

3 SRIF analogues displaced the binding of the four radioligands. $sst_{2/5}$ receptor-selective ligands showed much higher affinity than $sst_{1/3/4}$ receptor-selective ligands. The binding profile of [^{125}I]Tyr^3-octreotide was different from that of [^{125}I]LTT-SRIF-28, [^{125}I]CGP 23996 and [^{125}I]Tyr^{10}-cortistatin-14. The $sst_{5/1}$ receptor-selective ligand L-817,818 identified two binding sites, one with subnanomolar affinity (sst_5 receptors) and one with micromolar affinity (sst_2 receptors); however, the proportions were different: 70 – 80% high affinity with [^{125}I]LTT-SRIF-28, [^{125}I]CGP 23996, [^{125}I]Tyr^{10}-cortistatin-14, but only 20% with [^{125}I]Tyr^3-octreotide.

4 SRIF analogues inhibited the forskolin-stimulated cAMP levels depending on concentration. $sst_{2/5}$ receptor-selective ligands were highly potent, whereas $sst_{1/3/4}$ receptor-selective ligands had no significant effects. The sst_2 receptor antagonist D-Tyr^8-CYN 154806 competitively antagonised the effects of SRIF-14 and sst_2 receptor-preferring agonists, but not those of L-817,818.

5 The complex binding properties of SRIF receptor analogues indicate that sst_2 and sst_5 receptors are the predominant SRIF receptors expressed on AtT-20 cell membranes with no or only negligible presence of sst_1, sst_3 and sst_4 receptors. In the functional studies using cAMP accumulation, only sst_2 and sst_5 receptors appear to play a role. However, the 'predominant' receptor appears to be the sst_2 receptor, although sst_5 receptors can also mediate the effect, when the ligand is not able to activate sst_2 receptors. This clearly adds flexibility to SRIF-mediated functional effects and suggests that the physiological role of SRIF and its analogues may be mediated preferentially via one subtype over another.

British Journal of Pharmacology (2003) **139,** 109 – 121. doi:10.1038/sj.bjp.0705235

Keywords: Somatostatin; somatostatin receptor agonists; somatostatin receptor antagonists; AtT-20 mouse tumour corticotrophs; radioligand binding; cAMP measurements

Abbreviations: ACTH, adrenocorticotropin; DMEM, Dulbecco's MEM with Glutamax-I; EDTA, ethylenediaminetetraacetic acid; FRSK, forskolin; GH, growth hormone; HEPES, *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulphonic acid]; IBMX, isobutylmethylxanthine; SRIF, somatostatin, somatotropin release inhibitory factor; sst_1 – sst_5 receptors, SRIF receptor subtypes 1 – 5

Introduction

Somatostatin (SRIF; somatotropin release inhibitory factor) is known to be a potent regulator of endocrine secretion (Hannon *et al*., 2002a). In particular, in the anterior pituitary, SRIF acts as a neurohormone, secreted from neurones into the blood, and inhibiting the secretion of growth hormone (GH), adrenocorticotropin (ACTH) as well as other pituitary hormones. Biological actions of SRIF are exerted through the activation of multiple heptahelical, G-protein-linked SRIF receptor subtypes (sst_1 – sst_5) which are generally coupled to an inhibition of cAMP levels (Patel, 1999). SRIF receptors have a broad and overlapping tissue distribution; in particular, in the rat pituitary, sst_2 and sst_5 receptors are the principal subtype expressed (Mezey *et al*., 1998). To date, although numerous studies have explored the SRIF receptor function when expressed in transfected systems, assigning a pharmacological and functional profile to an individual native SRIF receptor remains difficult. This is due to the limited availability of

*Author for correspondence;
E-mail: daniel1.hoyer@pharma.novartis.com

selective agonists and antagonists with adequate pharmacokinetic properties. Furthermore, a mixed receptor subtype population in single cells may lead to confusion.

The AtT-20 mouse anterior pituitary tumour cell line has been widely used as a model to study the physiological functions of SRIF in corticotrophs. Indeed, in this cell line, SRIF is shown to inhibit ACTH secretion and GH mRNA levels (Sarret *et al*., 1999; Strowski *et al*., 2002). There is also evidence that SRIF induces apoptosis and decreases the interleukin-1 receptor density in AtT-20 (Takao *et al*., 1994; Srikant, 1995). AtT-20 cells are thought to endogenously express one or more subtypes of the SRIF receptor (Thermos & Reisine, 1988; Srikant *et al*., 1992). In particular, the presence of sst_1, sst_2, sst_4 and sst_5 receptors has been suggested from receptor mRNA studies, fluorescent immunolabelling, radioligand binding, cAMP accumulation assays, electrophysiological and hormone secretion studies (Patel *et al*., 1994; Hofland *et al*., 1995; Tallent *et al*., 1996b; Sarret *et al*., 1999; Strowski *et al*., 2002). In addition, the presence of a novel SRIF receptor on AtT-20 cell membranes has been suggested (Patel *et al*., 1994; Tallent *et al*., 1996a) although this has not been conclusively demonstrated using molecular biological tools. While such studies have proved useful, an exhaustive pharmacological characterisation of the SRIF receptors expressed in AtT-20 cell membranes does not exist. In particular, some discrepancies are apparent when comparing data from different groups (Patel *et al*., 1994; Sarret *et al*., 1999; Strowski *et al*., 2002), which may relate to the diversity of techniques and/or cell lines used, since the phenotype may change.

The objectives of the present study were (i) to use radioligand binding with different agonist radioligands to determine the expression levels of SRIF receptors and, if possible, the pharmacological signatures of native SRIF receptors in AtT-20 cells, and (ii) to investigate the functional effects of SRIF receptor activation on cAMP accumulation. SRIF receptors subtype-selective peptidyl and nonpeptidyl agonists as well as antagonists were used.

Methods

Cell culture

AtT-20/D16-16 clonal mouse tumour corticotrophs were a gift of Professor Bernard Koch (Université Louis Pasteur, Strasbourg, France). They were cultured in a complete medium composed of Dulbecco's MEM with Glutamax-I (DMEM) supplemented with 10% ($v\,v^{-1}$) foetal bovine serum, 100 UI ml^{-1} penicillin and 100 $\mu g\,ml^{-1}$ streptomycin at 37°C, 5% CO_2 and 95% relative humidity. For passaging, the cells were detached from the culture flask by washing with phosphate-buffered saline and brief incubation with trypsin/ethylenediaminetetraacetic acid (EDTA) (0.5 and 0.2 mg ml^{-1}, respectively). For crude cell membrane preparations, AtT-20 cells were scraped and centrifuged at 4°C for 5 min at 1000 × g. The cell pellet was either stored at − 80°C or used directly.

Radioligand binding assay

In saturation experiments, the cells were resuspended in binding assay buffer (0.5%. ($w\,v^{-1}$) bovine serum albumin, 10 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid] (HEPES, pH 7.5) by homogenisation with a Polytron homogeniser (Kinematica AG, Lucerne, Switzerland) at 50 Hz for 30 s. A volume of 150 μl of cell homogenate (ca. 1.0 – 2.5×10^4 cells) was then incubated with 50 μl of 12 different concentrations (2175 Ci $mmol^{-1}$, 5 – 500 pM final concentration) of [^{125}I]LTT-SRIF-28, [^{125}I]CGP 23996, [^{125}I]Tyr^{10}-cortistatin or [^{125}I]Tyr^3-octreotide in binding assay buffer containing $MgCl_2$ (5 mM) and the protease inhibitor bacitracin (20 $\mu g\,ml^{-1}$), and either 50 μl of binding assay buffer alone (total binding) or supplemented with 1 μM SRIF-14 (non specific binding).

In competition experiments, 150 μl of cell homogenate was incubated with 50 μl of [^{125}I]LTT-SRIF-28, [^{125}I]CGP 23996, [^{125}I]Tyr^{10}-cortistatin or [^{125}I]Tyr^3-octreotide (2175 Ci $mmol^{-1}$, 25 – 75 pM final concentration) in binding assay buffer containing $MgCl_2$ (5 mM) and bacitracin (20 $\mu g\,ml^{-1}$), and either 50 μl binding assay buffer alone (total binding) supplemented with 1 μM SRIF-14 (nonspecific binding) or with increasing concentrations of test compounds.

Experiments were conducted in triplicate. Incubation was terminated after 1 h at room temperature by vacuum filtration through glass fibre filters presoaked in 0.25% ($w\,v^{-1}$) 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 Packard TopCount (Packard Instruments Company, Meriden, CT, U.S.A.) using scintillation liquid (65% counting efficiency). Protein concentration was determined by means of the Bio-Rad Protein Assay Kit (Hemel, Hempstead, U.K.) using bovine serum albumin as a standard (Bradford, 1976).

Measurement of forskolin (FRSK)-stimulated cAMP accumulation

AtT-20 cells grown to ~80% of confluence in 24-well plates were incubated with 3 μCi of [8-^{3}H]adenine (25 Ci $mmol^{-1}$, 0.24 pM final concentration) in 500 μl 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) for 2 h at 37°C. Cells were then washed twice with assay buffer containing 1 mM isobutylmethylxanthine (IBMX) as a phosphodiesterase inhibitor. The cells were incubated at 37°C in 1 ml assay buffer containing IBMX in the absence (basal cAMP levels) and presence of FRSK (10 μM) with increasing concentrations of test compounds. Where D-Tyr^{8}CYN 154806 was used as antagonist, it was added 30 min before FRSK and the tested agonists. Experiments were conducted in duplicate. After 15 min, the medium was removed and replaced with 5% ($w\,v^{-1}$) trichloroacetic acid containing 100 μM ATP and 100 μM cAMP.

After 30 min at 4°C, [8-^{3}H]ATP and [8-^{3}H]cAMP were separated from the trichloroacetic acid extracts by sequential chromatography on Dowex AG 50W-X4 and Alumina columns (Bio-Rad). Dowex columns were washed with 10 ml H_2O, then loaded with cell extract and 3 ml H_2O. The eluate ([8-^{3}H]ATP) was measured in a β-scintillation counter. H_2O (8 ml) was loaded onto the Dowex columns, and the eluate loaded onto alumina columns that had been washed with 100 mM imidazole (10 ml), and the flow-through discarded. The [8-^{3}H]cAMP was eluted from the columns with 100 mM imidazole (6 ml) and measured in a Packard β-scintillation

counter. Dowex columns were regenerated with 2 M HCl (10 ml), the alumina columns with 1 M imidazole (5 ml). Basal values of cAMP were 0.02 ± 0.004 pmol well^{-1}. FRSK induced a ca. five- to 10-fold stimulation of cAMP levels. cAMP formation was calculated as the ratio cAMP (cAMP + ATP)$^{-1}$ and data points were calculated as a percentage of the FRSK response (= 100%).

Analysis of data

In radioligand binding experiments or cAMP accumulation measurements, pK_d, B_{max}, per cent high affinity site values, E_{max} (maximum percentage effect) and pEC_{50} (−log of the concentration producing half the maximum effect) were determined by nonlinear regression curve analysis of the concentration – effect responses using the computer programs ActivityBase and GraphPad Prism. Linear regression analysis was performed using GraphPad Prism and the correlation coefficient (r^2) was derived from this fit. The slope factor and pK_B estimates (−log of the estimated antagonist equilibrium dissociation constant) were determined by the Schild – Gaddum regression analysis. Where specified, results are given as means ± s.e.m. of the indicated n values. Means were compared by paired t-test analysis and where $P < 0.05$, differences were considered significant.

Chemicals

[^{125}I]LTT-SRIF-28, [^{125}I]CGP 23996, [^{125}I]Tyr^{10}-cortistatin-14 and [^{125}I]Tyr^3-octreotide (Siehler *et al*., 1999a) were custom synthesised from ANAWA AG (Wangen, Switzerland). [8-^{3}H]adenine was purchased from Amersham GmbH (Freiburg, Germany). SRIF-14 and SRIF-28 were purchased from Bachem AG (Bubendorf, Switzerland). CH-275, octreotide, Tyr^3-octreotide, BIM 23027, L-363,301, L-362,855, D-Tyr^8 CYN 154806 (Feuerbach *et al*., 2000; Nunn *et al*., 2002), L-797,591, L-796,778 (Rohrer *et al*., 1998), NNC 26-9100 (Liu *et al*., 1998), compounds 1–4 (Nunn *et al*., 2003b), compounds 6, 7a and 7e (Hay *et al*., 2001), SRA 880 (Hoyer *et al*., 2002), compound 4k (Poitout *et al*., 2001) were synthesised at Novartis Pharma AG (Basel, Switzerland). L-803,087 and L-817,818 (Rohrer *et al*., 1998) were kindly provided by Merck Research Laboratories (Rahway, NJ, U.S.A.). DMEM, foetal bovine serum, penicillin/streptomycin and trypsin/EDTA were purchased from Gibco BRL (Basel, Switzerland). Imidazole and bacitracin were purchased from Fluka AG (Buchs, Switzerland) and Serva GmbH & Co (Heidelberg, Germany), respectively. Where not specified, chemicals and reagents were obtained from Sigma-Aldrich Co (St. Louis, MO, U.S.A.).

Results

Saturation binding

[^{125}I]LTT-SRIF-28, [^{125}I]CGP 23996 and [^{125}I]Tyr^{10}-cortistatin-14 have been reported to bind with high affinity to sst_1 – sst_5 receptors, when expressed in recombinant systems. In contrast, [^{125}I]Tyr^3-octreotide labels only sst_2 and sst_5 receptors both with high affinity (Siehler *et al*., 1999a). The selectivity of [^{125}I]Tyr^3-octreotide for native sst_2 receptors has recently been further supported in our laboratory using autoradiographic

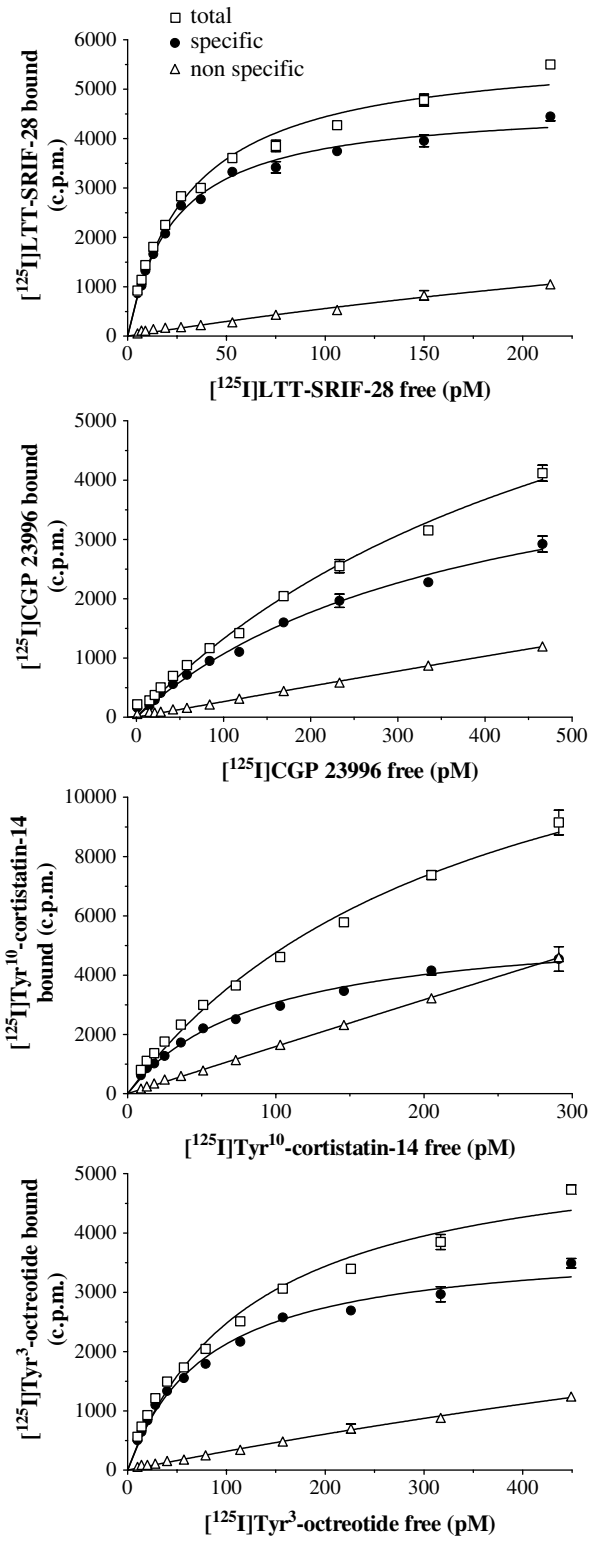


Figure 1 Saturation isotherms of [^{125}I]LTT-SRIF-28, [^{125}I]CGP 23996, [^{125}I]Tyr^{10} – cortistatin or [^{125}I]Tyr^3 -octreotide binding to crude membranes prepared from AtT-20 cells. The plots depict binding levels expressed as amount of radioligand bound vs. free radioligand concentration. The data points represent the mean ± s.e.m. (bars) of data from one example of three different experiments run in triplicate.

analysis of sst_2 receptor knockout mice brain (Hannon *et al*., 2002b), where [^{125}I]Tyr^3-octreotide binding was totally eliminated.

Saturation experiments show that [^{125}I]LTT-SRIF-28, [^{125}I]CGP 23996, [^{125}I]Tyr^{10}-cortistatin-14 and [^{125}I]Tyr^3-octreotide bind to AtT-20 cell membranes with high affinity (nanomolar) (Figure 1). Nonspecific binding was relatively low for all radioligands. In addition, the saturation isotherms were apparently monophasic suggesting that each radioligand is labelling a population of SRIF receptors for which it has equal affinity. However, different pK_d and B_{max} values were obtained (Table 1): [^{125}I]LTT-SRIF-28 and [^{125}I]CGP 23996 labelled a greater population (1.3- to 1.5-fold) of binding sites than [^{125}I]Tyr^{10}-cortistatin-14 and [^{125}I]Tyr^3-octreotide. [^{125}I]LTT-SRIF-28 had significantly higher affinity than the other three radioligands.

Table 1 Results of saturation experiments performed with different radioligands in AtT-20 cells

Radioligand	pK_d	B_{max}
[^{125}I]LTT-SRIF-28	10.68±0.06	315±16
[^{125}I]CGP 23996	9.40±0.02**	274±19
[^{125}I]Tyr^{10}-cortistatin-14	10.04±0.02**	239±11*
[^{125}I]Tyr^3-octreotide	9.98±0.10**	206±15*

The data are expressed as pK_d ($-\log M$) and B_{max} values (fmol mg^{-1}) ±s.e.m. of three different experiments. *$P<0.05$ and **$P<0.005$ vs [^{125}I]LTT-SRIF-28 values.

Competition binding

The pharmacological profiles of [^{125}I]LTT-SRIF-28, [^{125}I]CGP 23996, [^{125}I]Tyr^{10}-cortistatin-14 and [^{125}I]Tyr^3-octreotide-labelled sites were established in AtT-20 cell membranes using the native peptides SRIF-14, SRIF-28 and a number of synthetic peptidyl and nonpeptidyl agonists as well as antagonists which display different selectivities for SRIF receptor subtypes (Table 2, Figure 2). SRIF-28 bound with higher affinity than SRIF-14, except at [^{125}I]Tyr^3-octreotide-labelled sites where both peptides had equal affinity. $sst_{2/5}$ receptor-selective ligands displayed high to very high affinity (60 pM – 80 nM). The $sst_{5/1}$ receptor-selective ligand L-817,818 had apparently much lower affinity for the sites labelled by [^{125}I]Tyr^3-octreotide, compared to the other three radioligands. $sst_{1/3/4}$ receptor-selective ligands showed consistently very low affinity (300 nM – 300 μM) whichever radioligand was used. [^{125}I]LTT-SRIF-28, [^{125}I]CGP 23996 and [^{125}I]Tyr^{10}-cortistatin-14 binding profiles showed highly significant correlations (Figure 3) with relatively similar rank orders of potency: SRIF-28 > L-817,818 > SRIF-14 ⩾ octreotide > Tyr^3-octreotide > L-362,855 ⩾ BIM 23027 ⩾ compound 6 = L-363,301 > D-Tyr^8-CYN 154806 = compound 7a = compound 7e > L-797,591 > L-796,778 = CH 275 = L-803,087 ⩾ NNC 26-9100 > compound 4 = compound 4k > compound 1 = compound 2 > SRA-880 > compound 3 as defined by [^{125}I]LTT-SRIF-28. In contrast, [^{125}I]Tyr^3-octreotide-labelled sites displayed a somewhat different profile: BIM 23027 > SRIF-28 = SRIF-14 = Tyr^3-octreotide > octreotide = compound 6 > L-362,855 = L-363,301 > D-Tyr^8-CYN 154806 > compound 7e ⩾ compound 7a > L-817,818 > CH 275 = L-797,591 ⩾ compound 2 > L-796,778 > L-803,087 ⩾ NNC 26-9100 = compound 4 > compound 1 ⩾ SRA-880 = compound 4k > compound 3, as illustrated in Figure 3. In general, the peptides showed higher affinity for sites labelled with [^{125}I]CGP 23996 and [^{125}I]Tyr^{10}-cortistatin-14 when compared to [^{125}I]LTT-SRIF-28 (up to 5.5-fold). In addition, $sst_{2/5}$ receptor-selective ligands displayed higher affinity for sites labelled with [^{125}I]Tyr^3-octreotide when

Table 2 Comparison of SRIF receptor profiles defined with different radioligands in AtT- 20 cells

Ligand	*Reported selectivity*[a]	*[^{125}I]LTT- SRIF-28*	*[^{125}I]CGP 23996*	*[^{125}I]Tyr^{10} -cortistatin-14*	*[^{125}I]Tyr^3 –octreotide*
SRIF-28		9.89±0.05	9.83±0.09	9.72±0.13	9.90±0.03
SRIF-14		9.02±0.05	9.27±0.08*	9.22±0.03*	9.87±0.09**
L-817,818	$sst_{5/1}$ (agonist)	9.60±0.03	10.17±0.07**	10.00±0.02**	6.57±0.04**
Octreotide	$sst_{2/5}$ (agonist)	8.92±0.03	9.31±0.11*	9.22±0.04**	9.44±0.07**
Tyr^3-octreotide	$sst_{2/5}$ (agonist)	8.55±0.05	9.04±0.07*	9.06±0.14*	9.75±0.05**
L-362,855	$sst_{5/2}$ (agonist)	8.21±0.06	8.57±0.12	8.62±0.05*	8.74±0.03**
BIM 23027	sst_2 (agonist)	8.07±0.06	8.51±0.07*	8.53±0.06*	10.07±0.04**
Compound 6	sst_2 (agonist)	7.84±0.10	8.37±0.03*	8.43±0.04*	9.42±0.02**
L-363,301	sst_2 (agonist)	7.71±0.05	8.14±0.10*	7.91±0.13	8.69±0.09**
D-Tyr^8-CYN 154806	sst_2 (antagonist)	7.31±0.16	7.41±0.15	7.51±0.19	8.15±0.07*
Compound 7a	sst_2 (antagonist)	7.15±0.02	7.58±0.04**	7.57±0.04**	7.73±0.01**
Compound 7e	sst_2 (antagonist)	7.10±0.02	7.47±0.03**	7.51±0.03**	7.78±0.03**
L-797,591	sst_1 (agonist)	6.47±0.05	6.40±0.05	6.33±0.07	5.78±0.13*
L-796,778	sst_3 (agonist)	6.03±0.06	6.50±0.09*	6.33±0.02*	5.38±0.05**
CH- 275	$sst_{1/4}$ (agonist)	6.00±0.03	6.28±0.06*	6.02±0.03	5.87±0.07
L-803,087	sst_4 (agonist)	5.92±0.16	6.03±0.01	5.93±0.06	5.17±0.02*
NNC 26-9100	sst_4 (agonist)	5.68±0.07	6.09±0.04*	5.89±0.02*	5.04±0.05**
Compound 4	sst_4 (agonist)	5.42±0.05	6.16±0.06**	5.93±0.03**	5.00±0.04**
Compound 4k	sst_3 (antagonist)	5.42±0.04	5.64±0.07*	5.38±0.03	4.25±0.03**
Compound 1	sst_4 (agonist)	5.15±0.02	5.86±0.03**	5.58±0.05**	4.39±0.04**
Compound 2	sst_4 (agonist)	5.13±0.03	5.42±0.06*	5.37±0.03**	5.59±0.04**
SRA-880	sst_1 (antagonist)	4.64±0.02	4.58±0.06	4.07±0.07**	4.27±0.06**
Compound 3	sst_4 (agonist)	4.21±0.05	4.06±0.02	3.97±0.03*	3.59±0.21*

The data are expressed as pK_d values ($-\log M$) ±s.e.m. of at least three different experiments. *$P<$ 0.05 and **$P<0.005$ vs [^{125}I] LTT-SRIF- 28 values.

[a]From Rohrer *et al.* (1998), Patel (1999), Hay *et al.* (2001), Poitout *et al.* (2001), Hannon *et al.* (2002a), Hoyer *et al*. (2002), Nunn *et al.* (2003b).

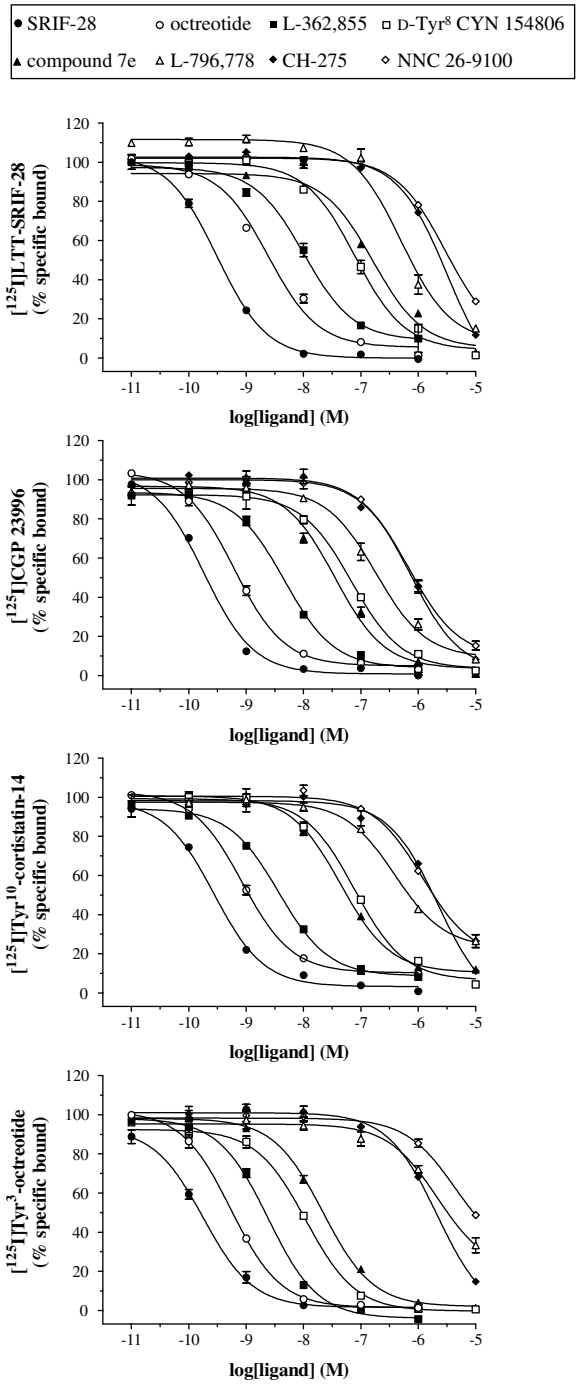


Figure 2 Inhibition of radioligand binding to AtT-20 cell membranes by SRIF analogues. Crude membrane preparations were incubated with [^{125}I]LTT-SRIF-28, [^{125}I]CGP 23996, [^{125}I]Tyr^{10}-cortistatin or [^{125}I]Tyr^{3}-octreotide, respectively, and the indicated concentrations of SRIF and SRIF receptor-selective ligands. Data are expressed as percentage of specific binding. The data points represent the mean ±s.e.m (bars) of data from one example of three different experiments run in triplicate.

compared to [^{125}I]LTT-SRIF-28, [^{125}I]CGP 23996 and [^{125}I]Tyr^{10}-cortistatin-14 (up to 100-, 36.3 and 34.6-fold, respectively) with the notable exception of L-817,818.

Competition curves for L-817,818 were then performed with 28 concentrations and analysed according to a two-site model (Table 3, Figure 4), since this ligand showed a rather divergent binding profile. At [^{125}I]LTT-SRIF-28-, [^{125}I]CGP 23996- and [^{125}I]Tyr^{10}-cortistatin-14-labelled sites; the high-affinity component represents the greater proportion of binding sites; in marked contrast, at [^{125}I]Tyr^{3}-octreotide-labelled sites, the high-affinity component represents only a very small proportion.

Functional assay: cAMP accumulation

The properties of SRIF receptors in AtT-20 cells were further investigated by measuring the effects of SRIF-14, SRIF-28 and synthetic analogues on FRSK-stimulated cAMP levels. As listed in Table 4, a concentration-dependent inhibition of cAMP accumulation was seen with most ligands and the rank order of potencies was: L-817,818 > BIM 23027 > SRIF-28 > SRIF-14 > Tyr^{3}-octreotide > compound 6 = octreotide > L-363,301 > L-362,855 ≥ compound 7a > compound 7e = compound 4k > L-796,778. $sst_{2/5}$ receptor-selective agonists showed full or close to full agonism with relative efficacies of 74 – 93% compared to that of SRIF-28, the most efficacious agonist (Figure 5). In contrast, the sst_3 receptor agonist L-796,778 had low potency and a relative efficacy of 46% at higher concentrations, at which it loses its selectivity (Rohrer *et al*., 1998). $sst_{1/4}$ receptor agonists did not produce significant inhibition of cAMP levels. sst_2 receptor antagonists displayed different effects (Figure 5), compounds 7a and 7e seemed to behave as effective agonists (76 and 85%, respectively) although with different potencies, whereas D-Tyr^{8}-CYN 154806 was totally devoid of intrinsic activity. In addition, the sst_3 receptor antagonist compound 4k showed a weak partial agonism (31%) only at high concentrations, while the sst_1 receptor antagonist SRA-880 did not produce significant effect. In agreement with previous results (Luini & De Matteis, 1990; Takao *et al*., 1994) SRIF-14 and SRIF-28 were largely devoid of effects on basal (in contrast to FRSK-stimulated) cAMP levels ($n = 3$; data not shown).

In another set of experiments, the concentration-dependent inhibition of FRSK-stimulated cAMP accumulation produced by SRIF-14, Tyr^{3}-octreotide, BIM 23027, compound 6 and L-817,818 was determined in the absence or presence of increasing concentrations of the sst_2 receptor antagonist D-Tyr^{8}-CYN 154806. As shown in Figure 6, D-Tyr^{8}-CYN 154806 produced a shift to the right in the concentration – response curve of SRIF-14, Tyr^{3}-octreotide, BIM 23027 and compound 6 without affecting the E_{max} observed. In contrast, D-Tyr^{8}-CYN 154806 displayed only weak antagonism when tested in the presence of L-817,818. As shown in Table 5, data processing with Schild – Gaddum analysis showed that D-Tyr^{8}-CYN 154806 behaves as a competitive antagonist (slope factor not different from unity) in the presence of SRIF-14, BIM 23027, compound 6 and Tyr^{3}-octreotide. However, when used as an antagonist against L-817,818, the slope factor is significantly different from unity ($P < 0.01$), suggesting that a simple competitive antagonism is not applicable in this case.

Comparison of radioligand binding with the inhibition of cAMP accumulation

The pharmacological profiles determined in radioligand binding and inhibition of FRSK-induced cAMP accumulation assays were compared. As illustrated in Figure 7, the potency of the tested compounds was relatively similar when compar-

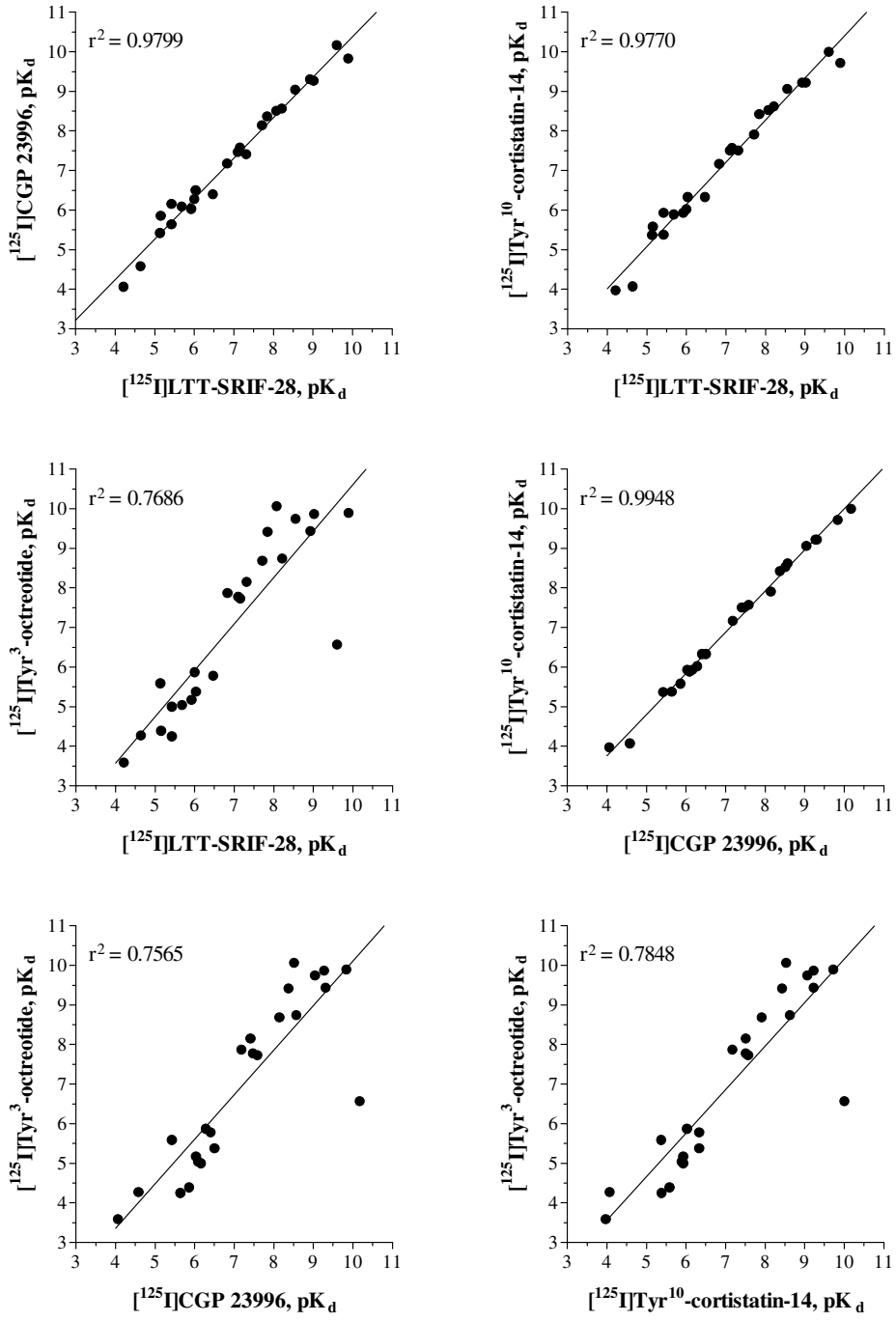


Figure 3 Comparisons of [^{125}I]LTT-SRIF-28, [^{125}I]CGP 23996, [^{125}I]Tyr^{10}-cortistatin and [^{125}I]Tyr^{3}-octreotide-defined pharmacological profiles in AtT-20 cells. Linear regression analysis was performed using the affinity values listed in Table 2. Correlation coefficients (r^2) are indicated in all the plots.

ing cAMP accumulation and radioligand binding with [^{125}I]LTT-SRIF-28, [^{125}I]CGP 23996 and [^{125}I]Tyr^{10}-cortistatin-14. In contrast, [^{125}I]Tyr^{3}-octreotide binding correlated poorly with cAMP accumulation.

Discussion

While different studies have shown that AtT-20 mouse corticotrophs endogenously express multiple SRIF receptors, a comprehensive SRIF receptor pharmacological characterisation has not been carried out. The present paper describes the expression levels, the pharmacological profiles with SRIF receptor subtype-selective ligands and the receptor – effector coupling of SRIF receptors in AtT-20 cells.

When considering saturation curves, they appear homogeneous with the four radioligands and the differences in B_{max} between [^{125}I]LTT-SRIF-28, [^{125}I]CGP 23996, [^{125}I]Tyr^{10}-cortistatin-14 and [^{125}I]Tyr^{3}-octreotide binding are very comparable to those seen in systems expressing homogeneous sst_2 or sst_5 receptor population (Siehler *et al*., 1999a, b; Feuerbach *et al*., 2000; Nunn *et al*., 2002). This would suggest that there is

Table 3 Two-site model analysis of the competition binding with the $sst_{5/1}$ receptor-selective agonist L-817,818 in AtT-20 cells

	L-817,818		
Radioligand	*p*K_d *(1)*	*p*K_d *(2)*	*% Sites (1)*
[^{125}I]LTT-SRIF-28	10.05±0.04	6.80±0.09	72.4±4.4
[^{125}I]CGP 23996	10.36±0.09	6.53±0.23	83.2±3.3
[^{125}I]Tyr^{10}-cortistatin-14	10.27±0.06	6.15±0.30	73.0±6.5
[^{125}I]Tyr^{3}-octreotide	11.06±0.22	6.24±0.10	20.2±4.7

The data are expressed as pK_d values ($-\log M \pm$s.e.m.) for the high (1) and low (2) affinity binding sites along with the number of high-affinity sites (per cent high-affinity sites± s.e.m.) of three different experiments.

little evidence for receptors other than sst_2 and sst_5 in these cells, since a significant density of sst_1, sst_3 or sst_4 receptors would have resulted in a significant increase in binding, as three of the four radioligands used ([^{125}I]LTT-SRIF-28, [^{125}I]CGP 23996, [^{125}I]Tyr^{10}-cortistatin-14) label sst_1 – sst_5 receptors with high affinity. The capacity of [^{125}I]LTT-SRIF-28 to label significantly more sites than [^{125}I]Tyr^{10}-cortistatin-14 and [^{125}I]Tyr^{3}-octreotide cannot be exclusively attributed to differences in receptor number, but is more likely to be because of properties of the radioligands, suggesting that the ligand – receptor complexes defined by each radioligand are conformationally different. In particular, it was shown that the radioligands may label different densities of sst_1 – sst_5 receptors as each of them seems to recognise different states (or combinations thereof) of the receptor, as for instance, G-protein coupled and uncoupled (Siehler *et al*., 1999a; Nunn *et al*., 2002). There have been rather different findings on SRIF receptor density in AtT-20 cells, with earlier studies (Patel *et al*., 1994; Hofland *et al*., 1995) demonstrating much higher expression levels than more recent studies (Sarret *et al*., 1999). Our own results appear to be in good agreement with the latter.

Competition experiments confirm that sst_1, sst_3 and sst_4 receptors are apparently not expressed in AtT-20 cells, since selective ligands for these receptors produced no significant effects except at higher concentrations at which they lose selectivity. sst_2 and sst_5 receptors are the predominant SRIF receptors present on AtT-20 cell membranes, as revealed by the high-affinity binding of selective ligands. These data are in agreement with previous radioligand binding studies, which revealed the presence of sst_2 and sst_5 receptors in AtT-20 cells (Patel *et al*., 1994; Hofland *et al*., 1995; Strowski *et al*., 2002). However, in studies using RNA extraction and Northern and Western blotting, a high degree of sst_2 receptor mRNA levels were also accompanied by low mRNA levels for sst_1 and sst_4 receptors. Low levels of a variant transcript of sst_5 receptors were also found (Patel *et al*., 1994). Recently, immunolabelling fluorescence experiments have shown the presence of sst_1, sst_2 and sst_5 receptors on AtT-20 cell membranes (Sarret *et al*., 1999). However, results obtained with different methodologies, which may not be as sensitive as radioligand binding studies, should be interpreted with caution. On the other hand, the contribution of sst_2 and sst_5 receptors is less obvious to explain. SRIF-28 bound to sites labelled by all four radioligands with higher affinity than SRIF-14, which may be indicative of a high population of sst_5 receptors. Indeed, sst_5 receptor is the only SRIF receptor showing (moderate)

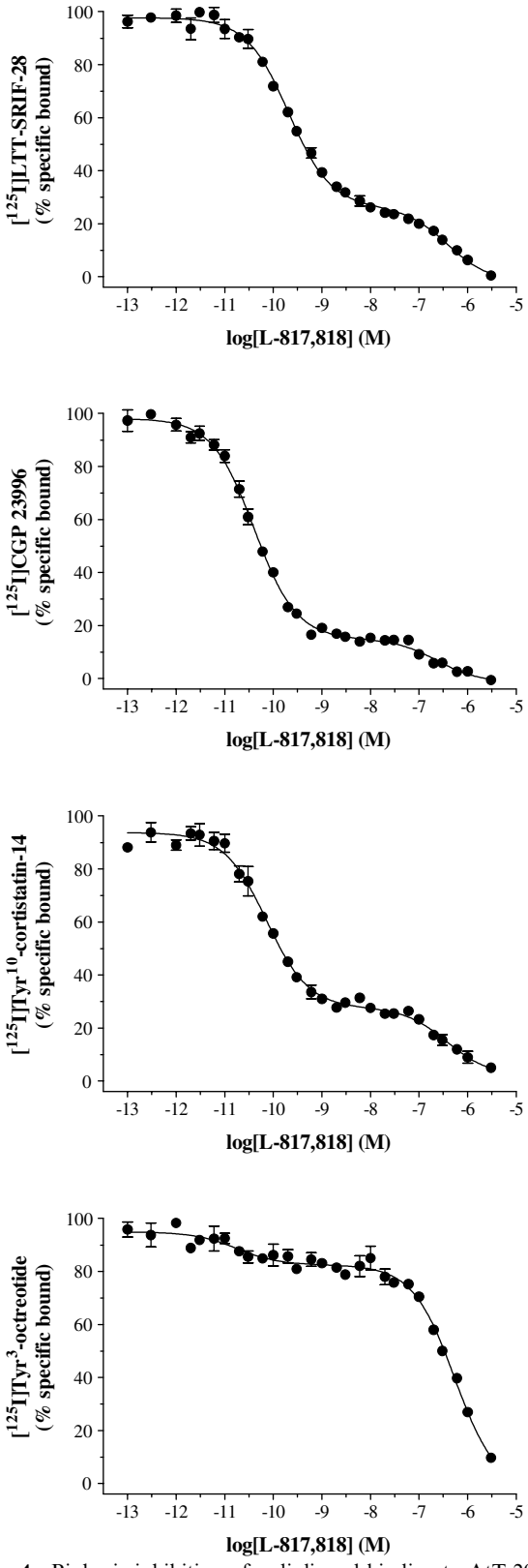


Figure 4 Biphasic inhibition of radioligand binding to AtT-20 cell membranes by the $sst_{5/1}$ receptor-selective compound L-817,818. Crude membrane preparations were incubated with [^{125}I]LTT-SRIF-28, [^{125}I]CGP 23996, [^{125}I]Tyr^{10}-cortistatin or [^{125}I]Tyr^{3}-octreotide, respectively, and the indicated concentrations of L-817,818. Data are expressed as percentage of specific binding. The data points represent the mean ±s.e.m. (bars) of data from one example of three different experiments run in triplicate. The curves were fitted according to a two-site model.

Table 4 Inhibition of FRSK-stimulated cAMP accumulation by SRIF and SRIF receptor-selective ligands in AtT-20 cells

Ligand	pEC_{50}	E_{max}
SRIF-28	9.56±0.17	64.8±3.07
SRIF-14	9.33±0.15	62.6±3.06
L-817,818	10.00±0.11	60.4±1.28
BIM 23027	9.71±0.12	52.5±2.06
Tyr^3-octreotide	9.22±0.11	60.3±1.93
Compound 6	8.97±0.22	50.4±2.64
Octreotide	8.96±0.17	59.6±3.38
L-363,301	8.54±0.19	47.8±3.02
L-362,855	7.92±0.11	59.9±4.00
Compound 7a	7.87±0.25	49.7±4.20
Compound 7e	6.61±0.16	55.2±4.60
Compound 4k	6.59±0.56	20.4±7.91
L-796,778	6.13±0.52	29.9±12.60
NNC 26-9100	—	11.5±4.00
D-Tyr^8-CYN 154806	—	11.1±2.20
CH-275	—	7.6±3.38
L-797,591	—	—
SRA-880	—	—
L-803,087	—	—
Compound 1	—	—
Compound 2	—	—
Compound 3	—	—
Compound 4	—	—

The data are listed as pEC_{50} ($-\log M$) and efficacy (E_{max}) on FRSK-induced cAMP accumulation ±s.e.m. of at least three different experiments plotted together.

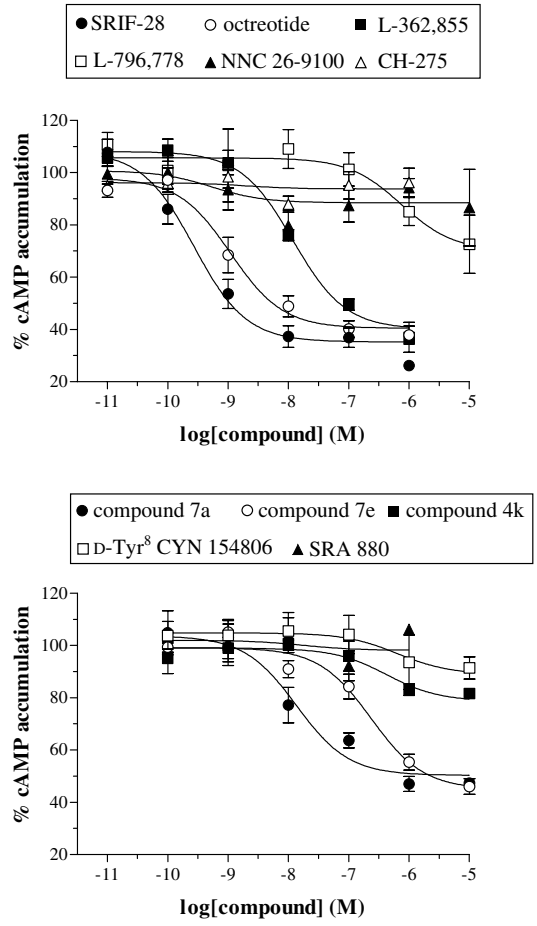
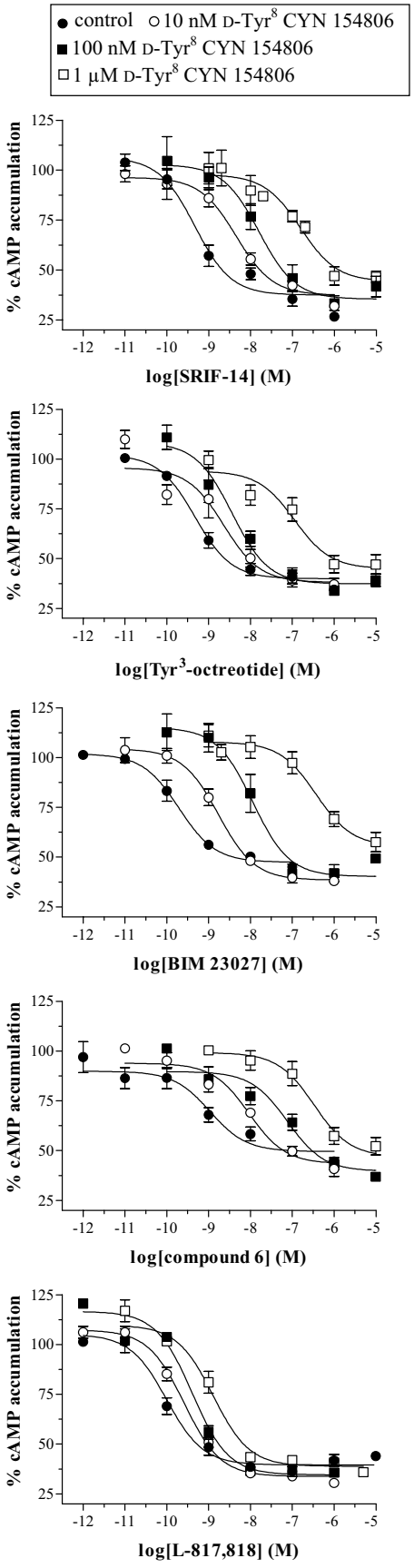


Figure 5 Inhibition of FRSK-stimulated cAMP accumulation by SRIF analogues in AtT-20 cells. Cells were treated with FRSK (10 μM) and the indicated concentrations of SRIF receptor-selective agonists (upper panel) and antagonists (lower panel). Graphs represent the percentage of inhibition of FRSK-stimulated cAMP accumulation. The data points represent the mean ±s.e.m. (bars) of data from one example of at least three different experiments run in duplicate.

preference for SRIF-28, at least when it is expressed in CHO cells (Williams *et al*., 1997). Previous experiments in AtT-20 cells have shown that sst_2 receptors account for only 38% of total binding sites (Patel *et al*., 1994). In addition, biphasic analysis of competition curves of the $sst_{5/1}$ receptor-selective agonist L-817,818 revealed that this compound binds to two different binding sites, one with high affinity and one with much lower affinity. It is likely that these binding sites correspond to sst_5 and sst_2 receptors respectively, both from the known binding affinities of the ligand for these receptors (Rohrer *et al*., 1998), and the fact that they exist when the radioligand used is [^{125}I]Tyr^3-octreotide. Interestingly, the population of sst_5 and sst_2 receptor sites is radioligand dependent, with sst_5 receptor sites being the greater population (70–80%) at [^{125}I]LTT-SRIF- 28-, [^{125}I]CGP 23996- and [^{125}I]Tyr^{10}-cortistatin-14-labelled sites, but only representing a very tiny proportion (20%) of the sites labelled by [^{125}I]Tyr^3-octreotide. This suggests that [^{125}I]Tyr^3-octreotide labels essentially sst_2 receptors, when the other three radioligands label essentially sst_5 receptors. This is not entirely expected, since if assuming that only sst_2 and sst_5 receptors are present in the cells, one would expect a similar proportion of both receptors whichever radioligand is being used, as all the four have nanomolar affinity for sst_2 and sst_5 receptors. Thus, it would make sense that all the four radioligands label a greater population of sst_5 receptors and a smaller population of sst_2 receptors or *vice versa*. We have shown previously in recombinant systems, [^{125}I]Tyr^3-octreotide labeling a much smaller population of sst_5 receptor binding sites than [^{125}I]LTT-SRIF-28 (15–20%), while it labelled similar densities at sst_2 receptors (Siehler *et al*., 1999a; Feuerbach *et al*., 2000). In mammalian brain, the binding profile of [^{125}I]Tyr^3-octreotide was not distinct from that of an sst_2 receptor (Schoeffter *et al*., 1995; Piwko *et al*., 1997a, b; Hannon *et al*., 2002b). However, other scenarios are possible to explain the biphasic curves of L-817,818 with different radioligand. For instance, SRIF receptors may assemble as homo- and heterodimers and dimerisation may then alter the pharmacological signature of these different receptor – ligand complexes. Indeed, it has been recently shown that some members of SRIF receptor family, including sst_2 and sst_5 receptors, undergo dimerisation even with other receptors (Rocheville *et al*., 2000a, b; Pfeiffer *et al*., 2001, 2002; Patel *et al*., 2002). AtT-20 mouse pituitary cells express endogenously several G-protein-coupled receptors, including muscarinic, substance P, interleukin-1 and ACTH-releasing factor receptors (Takao *et al*., 1994; Iredale & Duman, 1997; Kuzhikandathil & Oxford, 1999), no dopamine and opioid receptors, but the presence of dinorphine non-opioid receptors has been shown (Kuzhikandathil *et al*., 1998; Yarygin *et al*., 1998). Finally, we cannot rule out that the reason for ambiguities might be the use of cellular lysates for the binding experiments where receptors from intracellular stores may add further complexity. On the other hand, it was recently shown that on the whole AtT-20 cells[^{125}I]-SRIF bound is almost totally internalised after 20 min (Sarret *et al*.,



1999) and at all time points, the amount of [^{125}I]Tyr^3-octreotide internalised was much higher than the amount of radioligand that was membrane bound (Hofland *et al*., 1995) making difficult to perform a very thorough pharmacological characterisation of SRIF receptors in these conditions.

That the receptor populations labelled by the different radioligands are indeed different is confirmed by the pharmacological profile, where [^{125}I]LTT-SRIF-28, [^{125}I]CGP 23996, [^{125}I]Tyr^{10}-cortistatin-14 have overlapping profiles, which are somewhat different from that of [^{125}I]Tyr^3-octreotide: the latter is characteristic of sst_2 receptors, since the sst_2 receptor-preferring ligands have highest affinity, and the $sst_{5/1}$ receptor-selective L-817,818 the lowest, whereas SRIF-14 and SRIF-28 are equipotent. The profile of the other three radioligands is typically that of sst_5 receptors: L-817,818 has highest affinity, SRIF-28 is more potent than SRIF-14, and the sst_2 receptor-preferring ligands show somewhat lower affinity. Obviously, there is not a large difference of the profiles; but, this would be expected, since overall, most sst_2 receptor-selective compounds show also reasonable affinity for the sst_5 receptors, and there is only one ligand that sticks out, L-817,818. This is further illustrated by the one-to-one correlation between the binding profiles of [^{125}I]LTT-SRIF-28, [^{125}I]CGP 23996 and [^{125}I]Tyr^{10}-cortistatin-14: these correlated highly significantly, despite small variations in actual affinity values between radioligands, as has been reported in other models (Siehler *et al*., 1999a, b; Feuerbach *et al*., 2000; Nunn *et al*., 2002), suggesting that the overall population of SRIF receptors labelled by each radioligand is the same. In contrast, the binding profiles of [^{125}I]Tyr^3-octreotide were rather different in comparison to the other radioligands and therefore it is reasonable that there would be differences in profile between a ligand which mainly labels sst_2 receptors compared to those labelling mainly sst_5 receptors. Alternatively, we cannot rule out the possibility that ligands have different affinities for [^{125}I]Tyr^3-octreotide-labelled sites, since this radioligand has previously been shown to bind to a population of mainly G-protein-coupled receptors, in contrast to those of other radioligands that contain a mixed population of G-protein-coupled and-uncoupled receptors (Siehler *et al*., 1999a). In addition, examining the individual data, it appears that while $sst_{2/5}$ receptor-selective ligands do generally have higher affinity for [^{125}I]Tyr^3-octreotide-labelled sites than for sites labelled by [^{125}I]LTT-SRIF-28, [^{125}I]CGP 23996 and [^{125}I]Tyr^{10}-cortistatin-14, $sst_{1/3/4}$ receptor-selective ligands do not follow this pattern, demonstrating lower affinity for [^{125}I]Tyr^3-octreotide-labelled sites than for sites labelled by other radioligands. This may suggest that sst_1, sst_3 and/or sst_4 receptors are indeed present on AtT-20 cell membranes at least in very small numbers. Alternatively, we cannot exclude the possibility that a new SRIF receptor exists awaiting identification/cloning with a pharmacological profile

Figure 6 Antagonist activity of the sst_2 receptor-selective antagonist D-Tyr^8 CYN 154806 (30 min preincubation) on the inhibition of FRSK-stimulated cAMP accumulation induced by SRIF and SRIF receptor agonists in AtT-20 cells. Cells were treated with FRSK (10 μM), the indicated concentrations of SRIF-14, Tyr^3 -octreotide, BIM 23027, compound 6 and L-817,818, and either without or with increasing concentrations of D-Tyr^8 CYN 154806. Graphs represent the percentage of inhibition of FRSK-stimulated cAMP accumulation. The data points represent the mean $\pm$s.e.m. (bars) of data from one example of at least three different experiments run in duplicate.

Table 5 Parameters of antagonist properties of the sst_2 receptor-selective antagonist D-Tyr^8-CYN 154806 in the inhibition of FRSK-stimulated cAMP accumulation in AtT-20 cells

	D-Tyr^8-CYN 154806			
Ligand	*p*K$_B$ *(1)*	*p*K$_B$ *(2)*	*p*K$_B$ *(3)*	*Slope factor*
SRIF-14	8.94	8.52	8.49	0.77±0.11
Tyr^3-octreotide	8.44	7.70	8.27	0.91±0.38
BIM 23027	8.89	8.76	9.28	1.19±0.19
Compound6	8.88	8.85	8.49	0.80±0.09
L-817,818	8.21	7.48	7.07	0.43±0.09

The data are expressed as pK_B values ($-\log M$) for increasing antagonist concentrations ((1) 10^{-8} M; (2) 10^{-7} M; (3): 10^{-6} M) or slope factor ±s.e.m. from at least three different experiments plotted together.

different from any of the cloned SRIF receptors, as previously hypothesised (Patel *et al*., 1994; Tallent *et al*., 1996a).

Negative coupling of SRIF receptors to cAMP levels has been extensively demonstrated in different experimental models (Patel, 1999), including rat tumour somatotrophs (Petrucci *et al*., 2000; Cervia *et al*., 2002a, 2003). In AtT-20 cells, SRIF-14 has previously been shown to have no effect on basal levels of cAMP, while both SRIF-14 and SRIF-28 inhibited FRSK or corticotropin-releasing factor stimulated cAMP accumulation (Luini & De Matteis, 1990; Takao *et al*., 1994; Sarret *et al*., 1999; Strowski *et al*., 2002). This is further confirmed by our study, since both SRIF-14 and SRIF-28 potently and efficiently inhibited FRSK-induced cAMP accumulation, while they were devoid of effects on basal conditions. We cannot exclude that the lack of activity in the latter case is because of the fact that the values of basal cAMP accumulation obtained in our conditions are too low to detect any significant effect, although they are in the same range as in previous studies. Different effects of SRIF receptors on basal and FRSK-induced conditions have been shown in GC rat tumour somatotrophs (Cervia *et al*., 2003). In agreement with recent evidence (Strowski *et al*., 2002), in AtT-20 cells, the SRIF inhibitory effect on cAMP accumulation was apparently mediated via sst_2 and sst_5 receptors, as revealed by the full, or close to full agonism of $sst_{2/5}$ receptor-selective agonists. In particular, the potency of L-817,818 is high enough to exclude the possibility that its ability to inhibit cAMP accumulation is because of the activation of sst_2 receptors, and therefore proves the involvement of sst_5 receptors in this pathway. More likely, both sst_2 and sst_5 receptors are involved in the L-817,818 inhibition of cAMP accumulation. sst_1, sst_3 and sst_4 receptors appear to have no influence on cAMP accumulation in AtT-20 cells, since the selective ligands have not displayed agonism in the present studies.

At present, very few SRIF analogues have been shown to exhibit antagonistic activity with receptor subtype selectivity (Hay *et al*., 2001; Poitout *et al*., 2001; Cervia *et al*., 2002b; Hannon *et al*., 2002a). In addition, depending on the tissue type and receptor coupling efficiency, 'antagonists' may show agonism. In AtT-20 cells, D-Tyr^8-CYN 154806 had no agonist activity in the inhibition of FRSK-induced cAMP accumulation, while compounds 7a and 7e (which are also putative sst_2

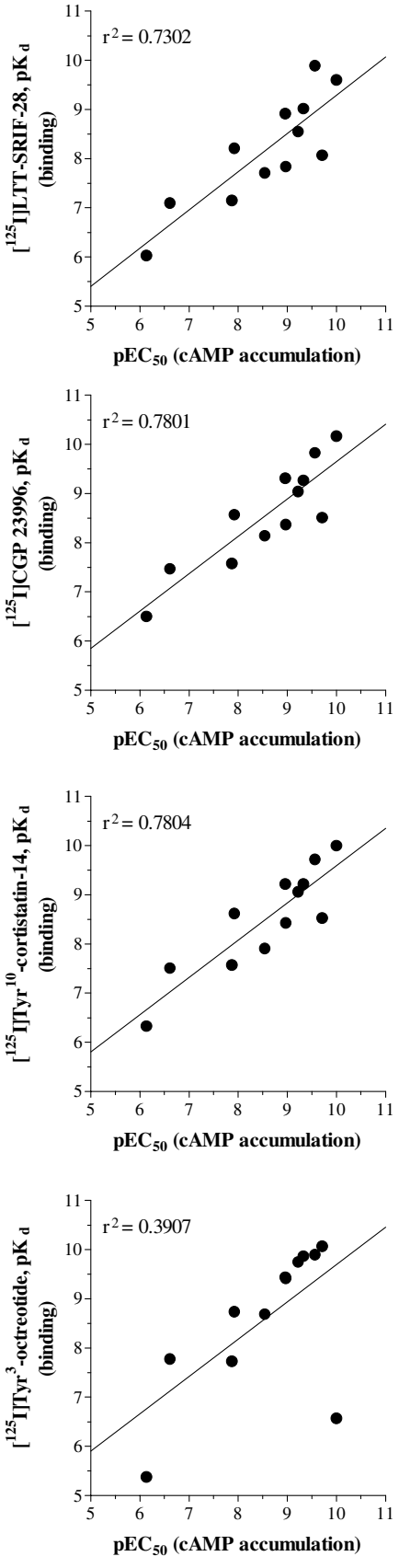


Figure 7 Comparisons of [^{125}I]LTT-SRIF-28, [^{125}I]CGP 23996, [^{125}I]Tyr^{10}-cortistatin and [^{125}I]Tyr^3-octreotide-defined pharmacological profiles and inhibition of FRSK-stimulated cAMP accumulation in AtT-20 cells. Linear regression analysis was performed using the affinity values listed in Tables 2 and 4. Correlation coefficients (r^2) are indicated in all the plots.

receptor antagonists) showed agonist properties. Previously, intrinsic activity has been shown for these three compounds in native and/or recombinant systems (Cervia *et al*., 2002b; Nunn *et al*., 2003a, c).

Antagonist studies with the sst_2 receptor-selective antagonist D-Tyr^8-CYN 154806 further confirm that the activation of sst_2 and sst_5 receptors inhibits cAMP accumulation in AtT-20 cells. D-Tyr^8-CYN 154806 potently and competitively antagonises the effects of SRIF-14, BIM 23027, compound 6 and Tyr^3-octreotide, suggesting that these agonists were acting primarily via sst_2 receptors, as if sst_5 receptors were not capable of acting on cAMP accumulation. However, further evidence that sst_5 receptors can also lead to inhibition of cAMP accumulation is provided by the low potency of D-Tyr^8-CYN 154806 to antagonise the $sst_{5/1}$ receptor-selective agonist L-817,818 effects (slope factor not compatible with unity, suggesting noncompetitive behaviour at sst_5 receptors). The weak antagonistic behaviour of D-Tyr^8-CYN 154806 can be reasonably explained by the fact that L-817,818 is also able, at least in part, to activate sst_2 receptors. Alternatively, D-Tyr^8-CYN 154806 antagonism may be related to its affinity at sst_5 receptors, as recently shown by Nunn *et al*. (2003c). Taken together, these data suggest that sst_2 and sst_5 receptors are redundant in their ability to control cAMP accumulation, since both receptors are capable of producing a maximal response alone. Again, the contribution of sst_2 and sst_5 receptors appears to be largely ligand-dependent, as if the different agonists may 'select' one receptor or another. In the present case, it would seem that the sst_2 receptor is 'dominant' since the compounds with sst_2 receptor preferring agonism (Tyr^3-octreotide, BIM 23027 and compound 6), or no receptor selectivity (SRIF-14), are equally well inhibited by the sst_2 receptor-selective antagonist D-Tyr^8-CYN 154806. This feature is interesting, and suggests that most compounds will act via sst_2 receptors, even if they are capable of acting at sst_5 receptors; it is only when sst_2 receptors cannot be activated as is the case with L-817,818, that the sst_5 receptor starts playing a significant and equivalent role. This is intriguing, since from the binding behaviour of the radioligands, of which the nonselective ones appear to label largely sst_5 receptors, the opposite behaviour may have been anticipated.

Finally, we compared the binding profiles of the four radioligands in competition studies with the ability of SRIF receptor agonists to inhibit cAMP accumulation. There is good correlation between affinities determined in binding studies and potencies in inhibition of FRSK-induced cAMP accumulation, suggesting that SRIF receptors expressed by AtT-20 cell membranes are also those involved in cAMP inhibition. The fact that [^{125}I]Tyr^3-octreotide binding correlated poorly can be easily explained by taking into account that L-817,818, by far the most potent in inhibiting cAMP accumulation, ranks 12th in binding, because of its binding to two different populations of SRIF receptors.

Concluding remarks

To date, the exact physiological role played by SRIF receptor activation in AtT-20 cells is partially known, but some points still remain obscure. sst_2 and sst_5 receptors seem to inhibit Ca^{2+} currents and cAMP levels, which in turn inhibits ACTH secretion (Tallent *et al*., 1996b; Strowski *et al*., 2002). Interestingly, SRIF is also able to inhibit ACTH secretion downstream of the control of Ca^{2+} entry and cAMP levels (Luini & De Matteis, 1990); however to date no information is available with respect to the receptor(s) involved, although the regulation of hormone release by sst_1 and sst_5 receptors, downstream of Ca^{2+} entry and cAMP levels was recently shown in GC rat tumour somatotrophs (Cervia *et al*., 2002b, 2003). In addition, in AtT-20 cells, SRIF receptors mediating SRIF effects on K^+ currents, interleukin-1 receptor density, apoptosis and GH mRNA levels remain to be established (Takao *et al*., 1994; Srikant, 1995; Tallent *et al*., 1996a; Sarret *et al*., 1999). Our study represents the first detailed and exhaustive pharmacological analysis of SRIF receptors in AtT-20 cells, which indicates the complex binding properties of some of the SRIF receptor analogues used. From binding studies, it seems clear that only sst_2 and sst_5 receptors are expressed; therefore, SRIF-induced effects in these cells are likely to be mediated by sst_2 and sst_5 receptors. As expected, in the functional studies using FRSK-stimulated cAMP accumulation, only these two receptors appear to play a role. However, to define the respective contribution of these two receptors, sst_2 and sst_5, turned out to be more complex than anticipated and the comparison of the binding and functional data is not straightforward. When using radioligand binding, it becomes clear that depending on the radioligand used, different receptor populations can be labelled, but at least as far as inhibition of cAMP accumulation is concerned, the 'predominant' receptor appears to be the sst_2 receptor, although sst_5 receptors can also mediate the effect, when the ligand is not able to activate sst_2 receptors. Thus, the presence of SRIF receptors is redundant in AtT-20 cells and it may well be that if another functional response was to be measured, another profile may be found, that is, one where the sst_5 receptor contribution is predominating. This clearly adds flexibility to SRIF-mediated functional effects and suggests that the physiological role of SRIF and its analogues may be mediated preferentially via one subtype over another.

This work was supported in part by EC contract QLG3-CT-1999-00908 and Swiss Grant BBW 00- 0427. We are grateful to Merck Research Laboratories (Rahway, NJ, USA) for providing us with the sst_4 and $sst_{5/1}$ receptor-selective nonpeptidyl ligands L-803,087 and L-817,818. We thank Dr Phillipe Schoeffter for the valuable discussions and advice.

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(Accepted February 12, 2003)