Dopamine receptor subtype 2 and somatostatin receptor subtype 5 expression influences somatostatin analogs effects on human somatotroph pituitary adenomas *in vitro*

M C Zatelli, D Piccin, F Tagliati, A Bottoni, M R Ambrosio, A Margutti, M Scanarini¹, M Bondanelli, M D Culler² and E C degli Uberti

Section of Endocrinology, Department of Biomedical Sciences and Advanced Therapies; University of Ferrara, Via Savonarola 9, 44100 Ferrara, Italy

¹Division of Neurosurgery, Hospital of Padova, 35100 Padova, Italy

²Biomeasure Incorporated/IPSEN, Milford, MA 01757-3650, USA

(Requests for offprints should be addressed to E C degli Uberti; Email: ti8@unife.it)

Abstract

Dopamine (DA) and somatostatin (SRIF) receptor agonists inhibit growth hormone (GH) secretion by pituitary adenomas. We investigated DA subtype 2 receptor (DR2) and SRIF receptor (sst) subtypes 2 and 5 expression in 25 GH-secreting pituitary adenomas and tested in primary culture the effects on GH and prolactin (PRL) secretion of sst agonists selectively interacting with sst2 (BIM-23120), sst5 (BIM-23206), and sst2 and sst5 (BIM-23244). All adenomas expressed sst2; eight adenomas expressed both sst5 and DR2, eight sst5 but not DR2, and eight DR2 but not sst5. One tissue lacked expression of DR2 and sst5. GH secretion was inhibited by BIM-23120 in all samples, while it was reduced by BIM-23206 only in adenomas not expressing DR2. BIM-23120's inhibitory effects correlated with sst2 and DR2 expression, whereas DR2 expression correlated inversely with BIM-23206 inhibitory effects on GH secretion. In seven mixed GH/PRL-secreting pituitary adenomas, PRL secretion was inhibited in sst5-expressing tumors by BIM-23206, but not by BIM-23120. BIM-23244 reduced PRL secretion only in adenomas expressing sst2, sst5 and DR2. sst5 and DR2 expression correlated directly with BIM23206 inhibitory effects on PRL secretion. Our results suggest that adenomas expressing DR2 are less likely to respond to clinically available SRIF analogs in terms of GH secretion inhibition. Therefore, drugs interacting also with DR2 might better control secretion of pituitary adenomas.

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Introduction

Somatostatin (SRIF) is known to inhibit growth hormone (GH) secretion by normal and neoplastic somatotroph cells, both *in vivo* (Melmed *et al.* 1998) and *in vitro* (Shimon *et al.* 1997), interacting with five distinct receptors (sst1–sst5), expressed at various levels in pituitary tumors (Patel 1999). Recently, pituitary sst expression has been shown to correlate with the inhibitory effects of SRIF analogs on GH secretion *in vitro* (Jaquet *et al.* 2000, Zatelli *et al.* 2003, Hofland *et al.* 2004). Moreover, a complex interplay between the different ssts is suggested by the evidence that G-protein-coupled receptors (GPCRs) form homo- and heterodimers (Rocheville *et al.* 2000a), supporting a greater functional diversity. This scenario is further complicated by the demonstration of functional dimerization between dopamine (DA) receptor subtype 2 (DR2) and sst5 *in vitro* (Rocheville *et al.* 2000b). DA interacts with a family of five GPCRs (DR1–DR5), and DR2 seems to be the more predominant subtype in the

anterior pituitary (Koga *et al.* 1987, Wood *et al.* 1991). DA agonists are the first choice for treatment of prolactin (PRL)-secreting pituitary adenomas and have been employed also in the treatment of GH-secreting pituitary adenomas, until SRIF analogs became available in clinical practice. Since DA attenuates GH secretion in acromegalic patients, DA agonists, including bromocriptine and cabergoline, have been used as either primary or adjuvant therapy for acromegaly (Vance *et al.* 1984) and cabergoline has been reported to suppress GH to less than 2 µg/l and to normalize insulin-like growth factor I in up to 30% of patients with acromegaly (Abs *et al.* 1998). DA agonists are preferred in acromegalic patients with coexistent hyperprolactinemia (Melmed *et al.* 2002), having an improved therapeutic efficacy when combined with SRIF analogs (Jaffe and Barkan 1992). Moreover, recent studies have shown that a chimeric molecule, interacting with both sst2 and DR2, has an enhanced potency in suppressing GH and PRL secretion from pituitary adenomas, suggesting an interaction between the two receptors

Table 1 Clinical data for acromegalic patients and *in vitro* GH and PRL secretion in pituitary adenomas

				<i>In vivo</i> (µg/l)			<i>In vitro</i> ^a (µg/l)	
No.	Sex	Age	Immuno-histochemistry	GH	IGF-I	PRL	GH	PRL
[sst2+, sst5+, DR2+] adenomas								
1 V	F	32	GH	101	1397	32.5	67.2	—
2	M	42	GH, PRL	100	870	3.3	12.8	—
3	F	42	GH	24.5	367.9	21.3	137.2	—
4	F	47	GH, PRL	46.7	876	14	24.7	—
5	F	38	GH	16	808	14.2	15.1	—
6	M	35	GH, PRL	19.5	928.7	10.4	111.5	—
7	F	57	GH, PRL	29	1000	48.7	40.1	6.9
8	F	25	GH, PRL	36.6	729	62.3	133.9	109.8
[sst2+, sst5+, DR2−] adenomas								
9	F	23	GH, PRL	20	1000	46.9	47.2	6.8
10	M	40	GH	10	1000	9.2	74.8	—
11	M	56	GH	3	287	9.8	5.4	—
12	M	42	GH	2.4	341	8.3	8.9	—
13	M	32	GH	20.8	528	12.7	3.2	—
14	F	42	GH	63	583	15.8	3.7	—
15	F	35	GH, PRL	19	650	56.7	173	4.4
16	F	81	GH	34.2	792.3	22.8	7.4	—
[sst2+, sst5−, DR2+] adenomas								
17	M	56	GH	9.6	439	6	89.7	—
18	F	49	GH, PRL	2.4	630	62	115.9	6.1
19	F	56	GH, PRL	8.9	615	45	77.3	6.8
20	M	53	GH	11.3	601	0.4	135	—
21	M	21	GH, PRL	6.92	766	40	238	44
22	F	44	GH	9.1	645	13	76.7	—
23	F	30	GH	14.4	901	1.75	96.6	—
24	M	57	GH	9.6	439	9	207	—
[sst2+, sst5−, DR2−] adenoma								
25	F	26	GH, PRL	26	950	62.5	157.9	7.2

^aGH and PRL levels (µg/l) in control culture medium from 12×10⁴ cells cultured for 12 h. IGF-I, insulin-like growth factor 1.

(Saveanu *et al.* 2002, Ren *et al.* 2003). However, it is unknown whether DR2 expression influences the effects of SRIF analogs on pituitary tumors.

We therefore investigated sst2, sst5 and DR2 expression pattern in somatotroph adenomas and tested in primary culture their response to SRIF-selective analogs interacting with sst2 and/or sst5, in terms of GH and PRL secretion inhibition.

Materials and methods

Human pituitary adenomas

25 patients, 10 males and 15 females, aged 42.4 ± 3.3 y (median, 42 y; range, 23–81 y) were diagnosed with acromegaly on the basis of clinical presentation, with pre-operative elevated serum GH (25.2 ± 5.5 µg/l, not declining to less than 1 µg/l during an oral glucose tolerance test) and insulin-like growth factor I levels (725.8 ± 52.6 µg/l) as expected for the age group, and

seller magnetic resonance imaging consistent with a pituitary macroadenoma (mean maximal diameter, 2.07 ± 0.8 cm; mean volume, 3.5 ± 0.7 cm³). Serum PRL was normal in 17 patients (12 ± 1.6 µg/l) and elevated to >30 µg/l in eight (53 ± 1.8 µg/l). Table 1 shows patients' characteristics and pre-operative hormonal values. All patients underwent trans-sphenoidal surgery, with sparing of the normal pituitary, and immunohistochemical examination for anterior pituitary hormones showed staining for GH in all cases, and also for PRL in 11 cases. Informed consent of the patients was obtained for disclosing clinical investigation and performing the *in vitro* study.

Tissue collection and primary culture

The collection and use of tumor samples were in accordance with the guidelines of the local committee on human research. Tissue samples were collected under sterile conditions at the time of surgery, and a fragment

was immediately frozen in liquid nitrogen and stored at -80°C until RNA isolation was performed. A portion of the fresh tissue was processed for primary culture, as described previously (Zatelli *et al.* 2003). Cells were resuspended in low-glucose Dulbecco's modified Eagle's medium with 10% fetal bovine serum and antibiotics (EuroClone, Wetherby, UK), seeded in 96-well culture plates and incubated at 37°C in a humidified atmosphere of 5% CO_2 /95% air. Medium was then changed to serum-free low-glucose Dulbecco's modified Eagle's medium containing 0.2% BSA, 120 nM transferrin, 5 U/l insulin, 2 mM glutamine and antibiotics, and cells were treated for 6 h with 10^{-8} M SRIF analogs. Medium was then collected and stored at -20°C for later hormone measurement. Fibroblast contamination was excluded by examining the morphology of cultured cells (Danila *et al.* 2001).

Isolation of RNA and reverse transcription (RT)-PCR

In order to demonstrate the pituitary origin of the samples, RT-PCR analysis for GH expression was performed in each specimen, and further expression analysis for sst2, sst5 and DR2 was performed only in GH-expressing tissues. Total RNA from the tumors was extracted with TRIzol reagent (Invitrogen, Milano, Italy) according to the manufacturer's protocol, and processed for RT-PCR analysis, as described previously (Zatelli *et al.* 2001). PCR conditions and oligonucleotide primers for amplification of GH, sst2 and sst5 were described previously (Zatelli *et al.* 2003). Amplification of DR2 cDNA (201 bp) was obtained by using the following primers and PCR conditions for 35 cycles: forward, 5'-CCCTT CATTGTCACCCTGCT-3'; reverse, 5'-CCTGTTCA CTGGAAACTCCC-3'. PCR conditions were as follows: denaturation at 95°C for 20 s, annealing at 55°C for 20 s and extension at 72°C for 30 s. PCR products were run on a 2% agarose gel, visualized by ethidium bromide staining and analyzed with the Fluor-S Multi Imager (Bio-Rad, Milano, Italy). To confirm the correct identification of RT-PCR products, their specificity was verified, after gel-purification by Quiaex II (Qiagen, Valencia, CA, USA), by restriction-enzyme digestion and direct sequencing (data not shown).

Quantitative PCR for sst2, sst5 and DR2 mRNAs

Quantitative PCR for sst2 and sst5 was performed as described previously (Zatelli *et al.* 2003, 2005), with primers and probes designed using Primer Express Software (Applied Biosystems, Monza, Italy). TaqMan probes (PE Applied Biosystems) labelled with a fluorescent dye (6-carboxy-fluorescein; FAM) and a quencher dye (6-carboxy-tetramethyl rhodamine; TAMRA) were used. For amplification of the house-keeping gene the Pre-Developed TaqMan Assay

Table 2 Human sst subtype specificity (IC_{50} , nM)

Compound	IC_{50} (nM)				
	sst 1	sst 2	sst 3	sst 4	sst 5
BIM-23120	1000	0.34	412	1000	213.5
BIM-23206	1152	166	1000	1618	2.4
BIM-23244	1020	0.29	133	>1000	0.67

Reagents for the Human 18 S rRNA ($20\times$) were used (PE Applied Biosystems).

Concerning DR2 amplification, Assay on Demand Hs00241436_m1 (Applied Biosystems) was used. Absolute quantitation of mRNA copy number in the samples was carried out following the Standard Curve Methods (Separate Tubes; User Bulletin no. 2 ABI PRISM 7700 Sequence Detection System; PE Applied Biosystems). Serial dilutions of the single-stranded sst2, sst5 and DR2 sense oligonucleotide amplicons (from 10^9 to 10^2 molecules) were carried out in triplicate. The log copy numbers of unknown samples were calculated from the regression line according to the equation: $\log N = (Ct - q)/m$, where Ct is the threshold cycle, q is the y intercept and m is the slope of the standard curve line. All quantitative PCRs were performed, recorded and analyzed using the ABI 7700 Prism Sequence Detection System. Slopes for all assays reported were -3.3 ± 0.1 .

All samples were carried out in triplicate (100 ng of reverse-transcribed total RNA per well) and repeated at least twice. For each sample one point of 18 S rRNA was loaded to evaluate the retro-transcription efficiency in the same plate and PCR conditions. A no-template control and RT controls were run in each experiment.

A cut-off of 3×10^3 mRNA copies/ μg total RNA was established as the threshold for real-time PCR to exclude the detection of transcripts due to illegitimate transcription, as suggested previously (Chelly *et al.* 1989, Korbonits *et al.* 2001).

sst-selective agonists

sst-selective agonists used in this study (BIM-23120, BIM-23206, BIM-23244) were provided by Biomeasure Incorporated/IPSEN (Milford, MA, USA) and their respective affinities to the different ssts are listed in Table 2. Specificity and selectivity of the analogs were determined by radioligand binding assay (Shimon *et al.* 1997, Saveanu *et al.* 2001), and biological activity of sst-selective agonists was evaluated as described by Shimon *et al.* (1997).

Hormone assay

To explore the effects of sst-selective agonists on pituitary primary cultures, human GH and PRL levels

were measured by immunoradiometric assay (IRMA) with reagents supplied by the Nichols Institute Diagnostics (San Juan Capistrano, CA, USA). The limit of detection for GH was 0.05 µg/l, with intra- and interassay variation coefficients of 3.3 and 6.1%, respectively. The limit of detection for PRL was 0.47 µg/l, with intra- and interassay variation coefficients of 6.4 and 5.9%, respectively. Hormone assays were performed in duplicate after appropriate sample dilutions of medium from cells treated for 6 h with each compound at 10^{-8} M (conditioned medium) in eight replicates for each pituitary adenoma.

Statistical analysis

Results are expressed as means $\pm$ S.E. Student's paired or unpaired *t*-test was used to evaluate individual differences between means. To measure the strength of association between pairs of variables without specifying dependencies, Spearman order correlations were run. A $P < 0.05$ was considered significant in all tests.

Results

SRIF and DA receptor mRNA expression

All tissue samples expressed GH, confirming their pituitary origin, and therefore each RNA was subjected to RT-PCR to investigate the expression of sst2, sst5 and DR2. We found that sst2 was expressed in all adenomas, sst5 in 16 and DR2 in 16. On the basis of sst and DR expression, four patterns were identified among the 25 tissues: eight adenomas expressed sst2, sst5 and DR2 ([sst2+, sst5+, DR2+] adenomas), eight expressed sst2 and sst5, but not DR2 ([sst2+, sst5+, DR2-] adenomas) and eight expressed sst2 and DR2, but not sst5 ([sst2+, sst5-, DR2+] adenomas). Only one adenoma lacked expression of both sst5 and DR2 ([sst2+, sst5-, DR2-] adenoma).

sst and DR expression were verified further by quantitative PCR (see Table 3). sst2 was expressed at almost the same mRNA level in [sst2+, sst5+, DR2+], [sst2+, sst5+, DR2-] and [sst2+, sst5-, DR2+] adenomas ($(6.7 \pm 1.3) \times 10^7$ molecules/µg reverse transcribed total RNA). Sample 25 (lacking expression of both sst5 and DR2) expressed higher sst2 mRNA levels (24×10^7 molecules/µg of reverse-transcribed total RNA). Moreover, real-time PCR confirmed the lack of sst5 expression in [sst2+, sst5-, DR2+] adenomas and in adenoma 25, and the lack of DR expression in [sst2+, sst5+, DR2-] adenomas and in adenoma 25 ($< 3 \times 10^3$ mRNA copies/µg reverse-transcribed total RNA). sst5 expression was > 3 fold higher in [sst2+, sst5+, DR2+] than in [sst2+, sst5+, DR2-] adenomas ($(1.1 \pm 0.2) \times 10^5$ compared with $(3.6 \pm 0.7) \times 10^4$ molecules/µg reverse-transcribed total RNA; $P < 0.01$). DR2 expression

was > 30 -fold higher in [sst2+, sst5+, DR2+] than in [sst2+, sst5-, DR2+] adenomas ($(7.8 \pm 1.6) \times 10^5$ compared with $(2.4 \pm 0.6) \times 10^4$ molecules/µg reverse-transcribed total RNA; $P < 0.01$).

Effects of selective SRIF analogs on GH secretion

All 25 pituitary primary cultures secreted measurable amounts of GH in the culture medium (see Table 1). Therefore, to investigate whether DR2 expression influences the effects of SRIF analogs on GH secretion *in vitro*, GH levels were measured in conditioned media from adenomas treated for 6 h with or without 10^{-8} M sst-selective agonists.

As shown in Fig. 1A and Table 3, we found that in [sst2+, sst5+, DR2+] adenomas treatment with the sst2-selective agonist (BIM-23120), with the combination of the sst2-selective agonist and the sst5-selective agonist (BIM-23120+BIM-23206) or with the sst2/sst5 dual agonist (BIM-23244) significantly inhibited GH secretion on average by 15–24% ($P < 0.05$), whereas treatment with the sst5-selective agonist (BIM-23206) did not modify the secretory activity of pituitary cells *in vitro* and did not potentiate the inhibitory effects of BIM-23120.

As shown in Fig. 1B and Table 3, in [sst2+, sst5+, DR2-] adenomas GH secretion was significantly reduced by treatment with BIM-23120 or BIM-23206 ($P < 0.05$), and even more so by treatment with BIM-23120+BIM-23206, or with BIM-23244 ($P < 0.01$; on average, 18–57%). An additive effect was indeed observed after co-treatment with the sst2- and sst5-selective agonists and after treatment with the dual sst2/sst5 agonist, which had a greater inhibitory effect on GH secretion as compared with treatment with BIM-23120 or with BIM-23206 alone ($P < 0.05$). Treatment with BIM-23206, with BIM-23206+BIM-23120 or with BIM-23244 were more effective in reducing GH secretion in [sst2+, sst5+, DR2-] adenomas as compared with [sst2+, sst5+, DR2+] adenomas ($P < 0.05$).

As shown in Fig. 1C and Table 3, in the eight [sst2+, sst5-, DR2+] adenomas GH secretion was significantly ($P < 0.05$) inhibited by BIM-23120, BIM-23120+BIM-23206 and BIM-23244 on average by 18–30%, while BIM-23206 alone had no effect. Treatment with each sst analog did not modify GH secretion by adenoma 25, which lacked expression of both sst5 and DR2.

Moreover, sst2 expression correlated directly with the inhibitory effects of BIM-23120 ($r^2 = 0.73$, $P < 0.01$) on GH secretion, whereas sst5 expression did not correlate with the inhibitory effects of BIM-23206. DR2 expression directly correlated with the inhibitory effects of BIM-23120 ($r^2 = 0.51$, $P < 0.05$), but correlated inversely with inhibitory effects of BIM-23206 ($r^2 = 0.52$, $P < 0.05$).

Table 3 sst2, sst5 and DR2 expression and *in vitro* GH and PRL secretion inhibition by sst-selective agonists in pituitary adenomas

mRNA expression (copies × 10 ⁶ /μg total RNA)			% GH secretion inhibition				% PRL secretion inhibition			
No.	sst2	sst5	DR2	BIM-23120	BIM-23206	BIM-23120+	BIM-23244	BIM-23120	BIM-23206	BIM-23120+
[sst2+, sst5+, DR2+] adenomas										
1	134.60	0.11	1.50	-44.0	22.9	-40.6	-40.1	n.d.	n.d.	n.d.
2	117.80	0.00	1.00	-42.9	17.3	-16.8	-22.0	n.d.	n.d.	n.d.
3	89.10	0.21	0.90	-25.4	-19.1	-31.3	-31.6	n.d.	n.d.	n.d.
4	38.50	0.08	0.70	-21.0	11.1	-3.8	-7.5	n.d.	n.d.	n.d.
5	52.30	0.13	0.80	-19.0	-7.3	-16.9	-14.9	n.d.	n.d.	n.d.
6	16.20	0.14	0.60	-10.9	-13.5	-11.7	-7.0	n.d.	n.d.	n.d.
7	5.10	0.08	0.03	-1.9	-5.9	6.8	1.7	-15.0	-33.5	-30.8
8	67.10	0.11	0.80	-24.4	9.7	-4.3	-6.8	-4.1	-53.5	-52.4
[sst2+, sst5+, DR2-] adenomas										
9	65.10	0.04	<0.003	-21.3	-0.8	-65.6	-52.2	-11.1	-12.0	-4.7
10	39.40	0.03	<0.003	-10.2	-34.7	-30.6	-34.6	n.d.	n.d.	n.d.
11	87.10	0.07	<0.003	-44.9	12.3	-67.7	-79.5	n.d.	n.d.	n.d.
12	57.10	0.03	<0.003	-24.9	-43.8	-51.4	-60.1	n.d.	n.d.	n.d.
13	66.00	0.05	<0.003	-28.7	-7.7	-36.3	-63.0	n.d.	n.d.	n.d.
14	57.00	0.004	<0.003	-24.8	-32.0	-37.3	-40.1	n.d.	n.d.	n.d.
15	72.00	0.04	<0.003	-31.3	-21.4	-46.0	-63.2	18.9	-35.0	-8.9
16	80.10	0.03	<0.003	-41.9	-13.4	-72.3	-67.3	n.d.	n.d.	n.d.
[sst2+, sst5-, DR2+] adenomas										
17	5.80	<0.003	0.01	-7.4	-9.6	-0.6	-1.5	n.d.	n.d.	n.d.
18	45.20	<0.003	0.02	-28.9	0.3	-20.8	-11.5	-6.2	10.4	5.1
19	321.80	<0.003	1.00	-71.4	33.0	-71.2	-61.8	4.4	-18.1	0.1
20	61.80	<0.003	0.40	-40.8	18.1	-14.5	-25.7	n.d.	n.d.	n.d.
21	56.80	<0.003	0.02	-33.7	0.2	-21.1	-14.4	6.7	13.4	3.7
22	7.20	<0.003	0.02	-11.0	0.4	-1.0	-2.8	n.d.	n.d.	n.d.
23	68.50	<0.003	0.04	-43.6	0.5	-29.4	-27.4	n.d.	n.d.	n.d.
24	5.80	<0.003	0.004	-2.4	-17.9	-3.6	-0.5	n.d.	n.d.	n.d.
[sst2+, sst5-, DR2-] adenoma										
25	241.10	<0.003	<0.003	-3.4	-1.7	-2.1	-2.7	1.4	0.9	-1.3

n.d., not determined.

Figure 1 Effects of BIM-23120, BIM-23206 and BIM-23244 on GH secretion in pituitary primary cultures. Pituitary primary cultures were treated with each SRIF analog, alone or in combination, at 10^{-8} M for 6 h and then GH concentration was assessed in the conditioned medium from eight replicates for each adenoma. GH secretion inhibition in primary cultures from (A) eight somatotroph [sst2+, sst5+, DR2+] adenomas, (B) eight somatotroph [sst2+, sst5+, DR2-] adenomas and (C) eight somatotroph [sst2+, sst5-, DR2+] adenomas. Results are expressed as means $\pm$ S.E. for GH suppression (%) in treated cells with reference to untreated control cells. * $P < 0.05$ and ** $P < 0.01$ versus untreated control cells; + $P < 0.05$ versus cells treated with BIM-23120 or BIM-23206.

Effects of selective SRIF analogs on PRL secretion

Of the 25 pituitary primary cultures, eight secreted measurable amounts of PRL in the culture medium (mixed GH-/PRL-secreting pituitary adenomas; see Table 1). In order to determine whether the effects of sst-selective agonists on PRL secretion are influenced by DR2 expression, PRL levels were measured in conditioned media from the eight mixed GH-/PRL-secreting pituitary adenomas treated for 6 h with 10^{-8} M sst-selective agonists.

As shown in Fig. 2A and Table 3, we found that in the two [sst2+, sst5+, DR2+] adenomas treatment with the sst2-selective agonist (BIM-23120) did not influence PRL secretion, which was significantly inhibited

Figure 2 Effects of BIM-23120, BIM-23206 and BIM-23244 on PRL secretion in pituitary primary cultures. Pituitary primary cultures from mixed GH-/PRL-secreting pituitary adenomas were treated with each SRIF analog, alone or in combination, at 10^{-8} M for 6 h and then PRL secretion was assessed in the conditioned medium from eight replicates for each adenoma. PRL secretion inhibition in primary cultures from (A) two mixed GH-/PRL-secreting [sst2+, sst5+, DR2+] adenomas, (B) two GH-/PRL-secreting [sst2+, sst5+, DR2-] adenomas and (C) three GH-/PRL-secreting [sst2+, sst5-, DR2+] adenomas. Results are expressed as means $\pm$ S.E. for PRL suppression (%) in treated cells with reference to untreated control cells. * $P < 0.05$ versus untreated control cells.

($P < 0.01$) by treatment with the sst5-selective agonist (BIM-23206), with the combination of the sst2-selective agonist and the sst5-selective agonist (BIM-23120 + BIM-23206) and with the sst2/sst5 dual agonist (BIM-23244) on average by 40–44%. No additive effect was apparent for BIM-23120 + BIM-23206 co-treatment, or for BIM-23244.

As shown in Fig. 2B and Table 3, in the two [sst2+, sst5+, DR2-] adenomas only treatment with BIM-23206 was effective in reducing PRL secretion (on average -23.5% ; $P < 0.05$). Moreover, treatment with BIM-23206 achieved a greater inhibition ($P < 0.05$) in PRL secretion in [sst2+, sst5+, DR2+] adenomas as compared with [sst2+, sst5+, DR2-] adenomas.

Furthermore, as shown in Fig. 2C and Table 3, none of the selective sst agonists had any effect on PRL secretion in the three [sst2+, sst5 -, DR2+] adenomas. PRL secretion by the [sst2+, sst5 -, DR2 -] adenoma (no. 25) was not modified by treatment with any selective sst agonist.

Moreover, the inhibitory effects of BIM-23206 correlated directly with sst5 and DR2 expression ($r^2=0.65$ and 0.52 , respectively; $P<0.05$).

Discussion

The demonstration of sst expression in human pituitary adenomas, even if highly heterogeneous (Nielsen *et al.* 1998), has provided several pharmacological tools for controlling hormonal hypersecretion by treatment with SRIF analogs (Hofland and Lamberts 2001). Moreover, DR expression has been documented extensively *in vivo* and *in vitro* in somatotroph adenomas with direct therapeutic implications in acromegalic patients (Koga *et al.* 1987, Ferone *et al.* 2001, Stefaneanu *et al.* 2001). Indeed, it has been shown that up to 30% of these patients are responsive to DA agonists (Abs *et al.* 1998), whereas in some cases dopaminergic agents are not effective, suggesting that DRs are not expressed or are less active (Bevan *et al.* 1989), since the absence of DR2 gene mutations in pituitary tumors has been demonstrated extensively (Friedman *et al.* 1994).

Our data demonstrate that sst and DR expression pattern may influence the antisecretory *in vitro* effects of SRIF analogs interacting with sst2 and/or sst5 in GH-secreting pituitary adenomas. According to previously published evidence (Shimon *et al.* 1997, Nielsen *et al.* 1998, Jaquet *et al.* 2000), in this study we found that sst2 is the most frequently expressed SRIF receptor subtype in GH-secreting adenomas. The crucial role for sst2 in the control of GH secretion is further substantiated by the demonstration that the sst2-selective agonist (BIM-23120) significantly inhibits GH secretion to the same extent in [sst2+, sst5+, DR2+] and [sst2+, sst5+, DR2 -] adenomas, as well as in [sst2+, sst5 -, DR2+] tissues. Moreover, the extent of GH secretion inhibition induced by the sst2-selective analog *in vitro* correlates significantly with sst2 expression. In this series of adenomas, sst2 mRNA levels are much higher than sst5 mRNA levels, in contrast with previous studies showing a prevalent sst5 expression in GH-secreting pituitary adenomas (Jaquet *et al.* 2000, Saveanu *et al.* 2001, Hofland *et al.* 2004). In our study we performed an absolute quantitation of sst mRNA molecules in each sample, avoiding the need for expression normalization against a housekeeping gene, which is questionable for real-time PCR purposes (Bustin 2000). Housekeeping-gene expression was indeed explored in order to evaluate RT efficiency. Moreover, we considered a

threshold of 3×10^3 mRNA copies/ μ g total RNA to discriminate between effective mRNA expression and transcripts due to illegitimate transcription, as shown previously (Bustin 2000). In our series 9 out of 25 GH-secreting pituitary adenomas had sst5 or DR2 expression level below the considered threshold and, on the basis of methodological considerations, were considered as not expressing sst5 and/or DR2. These findings differ from that reported by other authors, who showed sst5 and DR2 expression in all samples examined (Saveanu *et al.* 2001, 2002). These discrepancies might be due to methodological issues, related to normalization procedures followed by these authors.

sst5 and DR2 expression does not seem to influence BIM-23120 effects, suggesting that sst2 activation induces inhibitory effects on GH secretion independently of the expression of these two receptors. On the other hand, the sst5-selective agonist, BIM-23206, significantly inhibited GH secretion in [sst2+, sst5+, DR2 -] tissues, but not in [sst2+, sst5+, DR2+] adenomas, indicating that DR2 expression might characterize a group of adenomas that are resistant to treatment with sst5-selective agonists. This hypothesis is further strengthened by the finding that DR2 expression correlates inversely with the inhibitory effects of the sst5-selective agonist on GH secretion. Moreover, our data show a lack of correlation between sst5 expression levels and the inhibitory effect on GH secretion of the sst5-selective agonist. Therefore, it is not surprising to find that the latter is more effective in [sst2+, sst5+, DR2 -] adenomas, where sst5 is 3-fold less expressed than in [sst2+, sst5+, DR2+] adenomas. Moreover, the lack of efficacy of the sst5-selective agonist in [sst2+, sst5 -, DR2+] adenomas was in keeping with the absence of sst5 expression.

Treatment with the combination of sst2- and sst5-selective agonists (BIM-23120+BIM23206) obtained an additive effect in reducing GH secretion in [sst2+, sst5+, DR2 -] adenomas, but not in [sst2+, sst5+, DR2+], supporting the hypothesis that DR2 expression might characterize a subgroup of adenomas which are less responsive to sst5-selective agonists, and further indicating a possible negative interaction between sst5 and DR2 in the control of GH secretion. The lack of any additive effect of BIM-23120 and BIM-23206 on [sst2+, sst5 -, DR2+] adenomas was in keeping with the absence of sst5 expression. Treatment with the dual sst2/sst5 agonist (BIM-23244) obtained similar results, in line with previously reported evidence showing that the bi-selective analog can achieve greater GH suppression than sst2-preferential drugs (Saveanu *et al.* 2001).

In accordance with previous studies (Jaquet *et al.* 1999, Hofland *et al.* 2004) we found that sst5 activation is important for PRL secretion control, since the sst5-selective agonist (BIM-23206) significantly inhibited PRL secretion in both [sst2+, sst5+, DR2+] and [sst2+,

sst5+, DR2 –] mixed GH/PRL pituitary adenomas. Moreover, sst5 expression directly correlated with the inhibitory effects of BIM-23206 on PRL secretion. In keeping with this evidence, the sst5-selective agonist was more effective in [sst2+, sst5+, DR2+] adenomas, where sst5 mRNA expression is significantly greater than in [sst2+, sst5+, DR2 –]. These results might also indicate a possible positive interaction between sst5 and DR2 in controlling PRL secretion, contrary of that observed for GH. This hypothesis is strengthened further by the finding that DR2 expression correlates directly with the inhibitory effects of the sst5-selective agonist on PRL secretion. Moreover, the lack of efficacy of the sst5-selective agonist in [sst2+, sst5 –, DR2+] adenomas was in keeping with the absence of sst5 expression. On the other hand, the sst2-selective agonist failed to affect PRL secretion in each group, suggesting that sst2, at least in this subset of adenomas, does not contribute significantly to PRL secretion control, as also shown previously in other experimental settings (Jaquet *et al.* 2000). This hypothesis is further supported by the lack of any additive effect of the co-treatment with BIM23120+ BIM-23206 or with the bi-agonist BIM-23244 on PRL secretion. Our results are in keeping with previous evidence showing that the dual sst2/sst5 agonist, BIM-23244, and a sst5-selective agonist, BIM-23268, suppress PRL secretion in GH-/PRL-secreting pituitary adenomas with similar potency (Saveanu *et al.* 2001).

Our findings indicate that, in adenomas lacking DR2 expression, sst2 and sst5 agonists have an additive effect on GH secretion inhibition, which is not evident when DR2 is expressed. Therefore, our data indicate a negative interplay between sst5 and DR2 as far as GH secretion is concerned. On the other hand, inhibition of PRL secretion by the sst5-selective agonist is not enhanced by co-treatment with the sst2-selective agonist. Moreover, in DR2-expressing adenomas the sst5-selective agonist is nearly 2-fold more potent in inhibiting PRL secretion, suggesting a positive interplay between sst5 and DR2.

It has been shown recently that *in vitro* sst5 and DR2 associate on the plasma membrane and interact as functional oligomers (Rocheville *et al.* 2000b). Furthermore, sst5, existing in the basal state as a monomer upon activation by its ligand, forms sst5 oligomers and also heterodimerizes with sst1 (Rocheville *et al.* 2000a). Moreover, it has been shown that a hybrid molecule, BIM-23A387, with binding affinity for both sst2 and DR2, displays an enhanced potency in reducing GH secretion by pituitary adenomas (Saveanu *et al.* 2002). It is therefore possible that ssts and DRs assemble on the cell surface as oligomers and that the activation of a single receptor might have different effects depending on the components of the multi-receptor. Furthermore, as also suggested by our results, the activation of the same receptor complex might have different effects on

secretory activities (Lamberts *et al.* 2002). Moreover, our results suggest that DR2 expression characterizes a group of adenomas that are less likely to respond to clinically available SRIF analogs in terms of GH secretion inhibition *in vitro*. Therefore, drugs interacting also with DR2 might achieve better results in the control of secretion and growth of pituitary adenomas, as demonstrated by Saveanu *et al.* (2002).

In conclusion, our results show that sst and DR expression pattern influence the effects of SRIF analogs on human GH-secreting pituitary adenomas, supporting the hypothesis of an interaction between these receptors. Further research is needed to fully elucidate the complex interplay between GPCRs and the consequent biological effects, to identify suitable therapies controlling hormonal secretion of pituitary tumors.

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