EXPERIMENTAL STUDY

The multi-ligand somatostatin analogue SOM230 inhibits ACTH secretion by cultured human corticotroph adenomas via somatostatin receptor type 5

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Abstract

Objective: Currently, there is no effective medical treatment for patients with pituitary-dependent Cushing's disease. A novel somatostatin (SS) analogue, named SOM230, with high binding affinity to SS receptor subtypes sst_1, sst_2, sst_3 and sst_5 was recently introduced. We compared the *in vitro* effects of the sst_2-preferring SS analogue octreotide (OCT) and the multi-ligand SOM230 on ACTH release by human and mouse corticotroph tumour cells.

Methods: By quantitative RT-PCR the sst subtype expression level was determined in human corticotroph adenomas. *In vitro*, the inhibitory effect of OCT and SOM230 on ACTH release by dispersed human corticotroph adenoma cells and mouse AtT20 corticotroph adenoma cells was determined. In addition, the influence of dexamethasone on the responsiveness to OCT and SOM230 was studied.

Results: Corticotroph adenomas expressed predominantly sst_5 mRNA (six out of six adenomas), whereas sst_2 mRNA expression was detected at significantly lower levels. In a 72 h incubation with 10 nmol/l SOM230, ACTH release was inhibited in three out of five cultures (range -30 to -40%). Ten nmol/l OCT slightly inhibited ACTH release in only one of five cultures (-28%). In AtT20 cells, expressing sst_2, sst_3 and sst_5, SOM230 inhibited ACTH secretion with high potency (IC_{50} 0.2 nmol/l). Dexamethasone (10 nmol/l) pre-treatment did not influence the sensitivity of the cells to the inhibitory effect of SOM230, suggesting that sst_5 is relatively resistant to negative control by glucocorticoids.

Conclusions: The selective expression of sst_5 receptors in corticotroph adenomas and the preferential inhibition of ACTH release by human corticotroph adenoma cells by SOM230 *in vitro*, suggest that SOM230 may have potential in the treatment of patients with pituitary-dependent Cushing's disease.

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Introduction

The first choice of treatment of patients with pituitary-dependent Cushing's disease is surgery. If surgery fails, radiotherapy, alone or in combination with steroidogenic inhibitors, may be used (1, 2). These secondary options are primarily due to the absence of an effective medical treatment option (3, 4). Some preliminary data suggest a potential use of dopamine agonists, alone or in combination with ketoconazole, in selected cases of Cushing's disease or Nelson's syndrome (2). The expression of somatostatin (SS) receptors (sst) in adrenocorticotrophin (ACTH)-secreting pituitary adenomas has been studied *in vivo* and *in vitro*. *In vivo*, none of a series of eight corticotroph microadenomas showed

an increased uptake of ^{111}In-DTPA-labelled octreotide (OCT), whereas sst scintigraphy was positive in two invasive ACTH-secreting macroadenomas and two cases of Nelson's tumours (5). The current generation of sst_2-preferential SS analogues have no suppressive effect on ACTH level in patients with untreated Cushing's disease, who have elevated cortisol levels (6). However, the SS analogue OCT suppressed pathological ACTH release in patients with Nelson's syndrome and ACTH and cortisol secretion in patients with Cushing's syndrome caused by ectopic ACTH secretion (7–9). Overall, these data suggest that in corticotroph adenomas of untreated patients with Cushing's disease levels of sst_2, one of the five known sst subtypes to which OCT binds preferentially, are low, and that this receptor

subtype may be downregulated when circulating cortisol levels are high. The fact that ectopic ACTH-secreting tumours express sufficient sst numbers allowing their *in vivo* visualization by sst scintigraphy and responsiveness to OCT, even in the presence of a high cortisol level, can be explained by their relative resistance to glucocorticoids, compared with corticotroph adenomas (10). Additional *in vitro* evidence for a sst downregulation by hypercortisolaemia comes from studies using primary cultures of human corticotroph adenomas, in which it was shown that glucocorticoids downregulated the response of corticotrophin-releasing hormone (CRH)-induced ACTH secretion to OCT (11). Little is know yet about the quantitative expression levels of the five sst subtypes (sst_{1-5}) in normal and tumoral corticotroph cells. By double-labelling *in situ* hybridization analysis for sst_2 and sst_5 mRNA, Day *et al.* (12) showed that normal rat corticotrophs expressed preferentially sst_5 mRNA. In a few selected cases expression of sst subtype mRNA transcripts was studied in human corticotroph adenomas. In these studies it was found that the highest frequency of expression of sst mRNA transcripts was found for sst_2 and sst_5, and to a lesser extent sst_1 (13–16). Although sst_2 mRNA can be found in corticotroph adenomas, their levels of expression are apparently low since OCT has no effect on ACTH secretion in patients with pituitary-dependent Cushing's disease. However, at present the role of the other frequently expressed sst subtype, sst_5, on ACTH secretion by corticotroph adenoma cells is unclear.

Recently, a novel multi-ligand SS analogue, SOM230, has been synthesized. Compared with OCT, SOM230 has a 30-, 5- and 40-times higher binding affinity for sst_1, sst_3 and sst_5 receptors respectively, and 2.5-times lower affinity for sst_2 (17). The very high affinity of this SS analogue for sst_5 probably forms the basis for the higher potency of SOM230 in reducing insulin-like growth factor-I concentrations in rats, primates and dogs (18). Moreover, the very favourable elimination half-life of SOM230, which is 23 h (17), makes this novel compound suitable for clinical application as well.

The present study was carried out to further explore the potential functional significance of sst subtypes expressed in human corticotroph adenomas. We evaluated the effect of the SS analogue SOM230 on ACTH secretion by primary cultures of human corticotroph adenomas and mouse AtT20 corticotroph tumour cells.

Materials and methods

Patients

Pituitary tumour samples were obtained by transsphenoidal operation from 11 patients with Cushing's disease due a corticotroph adenoma as described in detail previously (19). ACTH-dependent hypercortisolism was biochemically established by the absence of cortisol diurnal rhythm, an increased 24 h free cortisol excretion, insufficient suppression of plasma cortisol concentrations after administration of 1 mg dexamethasone (DEX) and normal to increased plasma ACTH concentrations. Magnetic resonance imaging of the pituitary showed a microadenoma in seven patients, a macroadenoma in one patient and no adenoma in three patients. In these latter patients, pituitary ACTH overproduction was determined by sinus petrosus inferior sampling. Histological examination showed adenomatous tissue ($n = 9$; case no. 1 and nos 4–11) or hyperplasia ($n = 2$; case nos 2 and 3) with immunohistochemically expression of ACTH. All patients gave their informed consent for the use of tumour material for research purposes. After surgery, 7 of 11 patients were cured.

In six cases (nos 1–6) the tissue was directly snap-frozen on dry ice and stored at $-80\,^{\circ}C$ until analysis. Adenoma tissue from the five additional patients (culture nos 7–11) was directly used for cell culturing.

Quantitative PCR

Quantitative PCR was performed as described previously (20). Briefly, poly A^+ mRNA was isolated from adenoma tissue using Dynabeads Oligo (dT)25 (Dynal AS, Oslo, Norway). cDNA was synthesized using the poly A^+ mRNA, which was eluted from the beads in 40 μl H_2O for 2 min at 65 °C, using Oligo (dT)12–18 Primer (Invitrogen). One-twentieth of the cDNA library was used for quantification of sst subtype mRNA levels. A quantitative PCR was performed by TaqMan Gold nuclease assay (Perkin Elmer Corporation, Foster City, CA, USA) and the ABI PRISM 7700 sequence Detection System (Perkin Elmer, Groningen, The Netherlands) for real-time amplifications, according to manufacturer's protocol. The assay was performed using 15 μl TaqMan Universal PCR Master Mix (Applied Biosystems, Alphen a/d Rÿn, The Netherlands), 500 nmol/l forward primer, 500 nmol/l reverse primer, 100 nmol/l probe and 10 μl cDNA template, in a total reaction volume of 25 μl. After an initial heating at 95 °C for 8 min, samples were subjected to 40 cycles of denaturation at 95 °C for 15 s and annealing for 1 min at 60 °C. The primer and probe sequences that were used are indicated below. The detection of hypoxanthine-phosphoribosyl-transferase (HPRT) mRNA served as a control and was used for normalization of the sst subtype mRNA levels.

The primer and probe sequences that were used for the detection of sst_1, sst_2, sst_3, sst_5 and HPRT mRNAs have been described previously (20). In addition to these primers and probes, we also evaluated sst_4 mRNA expression in the present study using the following primers and probe: sst_4 forward 5′-CTGCGCC-AACCCTATTCTCT-3′; sst_4 reverse 5′-ACCCGCTGGAAG-GATCG-3′; sst_4 probe 5′-FAM-TGGCTTCCTCTCCGACA-

ACTTCCG-TAMRA-3′. Primers and probes were purchased from Biosource (Nivelles, Belgium).

The relative amount of sst subtype mRNA was determined using a standard curve generated from known amounts of human genomic DNA. For determination of the amount of HPRT mRNA, a standard curve was generated of a pool of cDNAs from a human cell line known to express HPRT. The relative amount of sst subtype mRNA was calculated relative to the amount of HPRT mRNA and is given in arbitrary units. Each sample was assayed in duplicate. Poly A^+ mRNA from AtT20/D16V cells was isolated as described above. PCR analysis to determine the expression of mouse sst_{1-5} mRNAs was performed as described elsewhere (21).

Cell dispersion and cell culture

Pituitary adenoma tissue Single cell suspensions of the pituitary adenoma tissues were prepared by enzymatic dissociation with dispase as described in detail previously (19). For short-term incubation of monolayer cultures, the dissociated cells were plated in 48-well plates (Corning BV Life Sciences, Schiphol-Rijk, The Netherlands) at a density between 10 000 and 50 000 cells per well per 1 ml culture medium. After 3–4 days the medium was changed and 4, 24 or 72 h incubations without or with test substances were initiated. At the end of the incubation the medium was collected and stored at −20 °C until hormone determination. The cells were cultured at 37 °C in a CO_2 incubator. The culture medium consisted of Minimum Essential Medium with Earle's salts supplemented with non-essential amino acids, sodium pyruvate (1 mmol/l), 10% fetal calf serum (FCS), penicillin (1×10^5 U/l), Fungizone (0.5 mg/l), L-glutamine (2 mmol/l), and sodium bicarbonate (2.2 g/l), pH 7.6. Media and supplements were from Invitrogen. Unfortunately, not enough tumour material was obtained to test the adenoma cells at all time-points and the dose-dependency of the effects by the indicated drugs.

Mouse corticotroph adenoma cells AtT20/D16V mouse corticotroph tumour cells (Dr J Tooze, European Organization for Molecular Biology) were routinely passaged by trypsinization as described in detail previously (22). The cells were maintained in 75 cm^2 culture flasks in Dulbecco's Minimal Essential Medium, supplemented with non-essential amino acids, sodium pyruvate (1 mmol/l), 10% FCS, penicillin (1×10^5 U/l), Fungizone (0.5 mg/l), L-glutamine (2 mmol/l) and sodium bicarbonate (2.2 g/l), pH 7.6. For experiments, the cells were seeded at a density of 20 000 cells per well in 1 ml of culture medium. After 72 h, the medium was changed and 4, 24 or 72 h incubations without or with OCT, SOM230 or SS-14 were initiated. In addition, the acute effect of SS-14, OCT and SOM230 was evaluated in a 3 h incubation without or with

10 nmol/l CRH. In order to evaluate the effect of glucocorticoids on SS analogue-induced inhibition of ACTH release, the cells were pre-treated in some experiments for 48 h with the synthetic glucocorticoid DEX (10 nmol/l). Thereafter, the medium was changed and a 3 h incubation without or with OCT (1 nmol/l) or SOM230 (1 nmol/l) in the presence of CRH (10 nmol/l) was performed. After the indicated time periods, the medium was collected and stored at −20 °C until determination of ACTH concentrations. For determining the effects of the compounds on cell growth, [^{3}H]thymidine incorporation, as well as the DNA content of the wells, was measured as described in detail elsewhere (23).

Hormone determinations

Human ACTH concentrations were determined by a non-isotopic, automatic chemiluminescence immunoassay system (Immulite; DPC Inc., Los Angeles, CA, USA). Intra- and interassay coefficient of variation (CV) values were 5.6 and 7.8% respectively. Human growth hormone (GH), prolactin (PRL), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) concentrations in the media were determined, in order to exclude the presence of contaminating normal pituitary cells in the cultures. GH, PRL, LH and FSH concentrations were determined by a non-isotopic, automatic chemiluminescence immunoassay system as well (Immulite; DPC). Intra- and interassay CV values for GH, PRL, LH and FSH were 6.0, 5.7, 5.7 and 6.4% and 6.2, 6.4, 12.3 and 7.5% respectively. Except for the expected hormone ACTH, none of the other hormones was detected (not shown).

Test substances

OCT (Sandostatin) was obtained from Novartis Pharma AG (Basle, Switzerland). SOM230 was a gift from Novartis Pharma AG. SS-14 was purchased from Sigma Chemical Co. Binding affinities of SS-14, OCT and SOM230 to the five sst subtypes are shown in Table 1. DEX was obtained from the Erasmus MC Pharmacy (Rotterdam, The Netherlands). CRH was purchased from Ferring (Hoofddorp, The Netherlands).

Statistical analysis of the data

All data on hormone release are expressed as means± S.E., $n = 4$ wells per treatment group. All data were analysed by ANOVA to determine overall differences between treatment groups. When significant differences were found by ANOVA, a multiple comparison between treatment groups was made using the Newman–Keuls test. Calculation of IC_{50} values for inhibition of hormone release were made using Graph-Pad Prism (San Diego, CA, USA).

Table 1 Binding affinity of SS-14, OCT and SOM230 for the five human sst subtypes, sst_{1-5}. Data are reproduced with permission from the society of the European Journal of Endocrinology (17). Results are mean±S.E. IC_{50} values (nmol/l).

Compound	$hsst_1$	$hsst_2$	$hsst_3$	$hsst_4$	$hsst_5$
SS-14	0.93±0.12	0.15±0.02	0.56±0.17	1.5±0.4	0.29±0.04
OCT	280±80	0.38±0.08	7.1±1.4	>1000	6.3±1.0
SOM230	9.3±0.1	1.0±0.1	1.5±0.3	>100	0.16±0.01

Results

sst subtype mRNA expression

In six out six human corticotroph adenomas (nos 1–6), sst_2 mRNA was detectable. However, the relative copy numbers were low (varying between 8 and 141 copies/HPRT). The relative sst_2 copy number was considerably lower compared with the majority of GH-secreting pituitary adenomas, as recently reported (20). sst_5 mRNA was detectable at much higher levels in six out of six adenomas (relative copy number between 277 and 1217). In the two cases in which histological analysis of the tissue revealed hyperplasia of ACTH-producing cells (case nos 2 and 3), sst_5 mRNA levels were relatively high. On the other hand, one ACTH-secreting pituitary adenoma (case no. 1) expressed the highest level of sst_5 mRNA in the series. sst_1, sst_3 and sst_4 mRNA was detectable at low levels in only one, two and two out of six samples respectively (Fig. 1).

In agreement with the results of RT-PCR analysis of the corticotroph adenomas, the sst subtype mRNAs expressed in ACTH-secreting mouse AtT20 corticotroph pituitary tumour cells were sst_2, sst_3 (low abundance) and sst_5 (Fig. 2).

Effect of SS-14, OCT and SOM230 on basal and CRH-induced ACTH secretion by primary corticotroph adenoma cells

Since not sufficient tissue was obtained to carry out both mRNA and culture studies on the same tissue, cell culture experiments were performed using adenoma tissue from five additional patients (nos 7–11). In a 72 h incubation, SOM230 (10 nmol/l) significantly suppressed ACTH secretion in three out of five primary cultures of human corticotroph adenomas (between 30 and 40% suppression). In contrast, OCT (10 nmol/l) suppressed basal ACTH release in only one out of five cultures (28% suppression) (Fig. 3A and B). From one corticotroph adenoma (no. 8) sufficient cells were obtained to perform a time-course study of the effects of SS-14, OCT and SOM230 on basal ACTH release. As shown in Fig. 4A (4, 24 and 72 h incubation), statistically significant ($P < 0.01$ vs untreated control cells) suppression of ACTH release was observed only after 72 h of incubation with SOM230 (10 nmol/l) and SS-14 (10 nmol/l). Ten nmol/l of OCT did not

significantly inhibit basal ACTH release at any time-point. A comparable time-course experiment was performed in mouse AtT20 cells. In agreement with the observations in primary human corticotroph adenoma cultures, SOM230 and SS-14, but not OCT, all tested at 10 nmol/l, suppressed basal ACTH production (Fig. 4B). The effect of SOM230 on basal ACTH release by AtT20 cells was dose-dependent with an IC_{50} value of 0.2 nmol/l (Fig. 5A), corresponding to the binding affinity of SOM230 for sst_5. In contrast, OCT inhibited ACTH release with much lower potency with a slight suppression at 100 nmol/l (Fig. 5A). Taken together, these data suggest that the effect of SOM230 on basal ACTH release is most probably mediated via sst_5. OCT and SOM230 did not inhibit AtT20 cell proliferation as measured by [³H]thymidine incorporation (Fig. 5B), and had no effect on the DNA content of the cells (not shown).

In contrast to the absence of an effect of OCT on basal ACTH release by AtT20 cells, OCT (10 nmol/l) significantly suppressed CRH (10 nmol/l)-induced ACTH release by 52% and SS-14 by 47% in a 3 h incubation. SOM230 was significantly more potent and inhibited CRH-induced ACTH release by 76% (Fig. 6). The effect of SS-14 on CRH-induced ACTH-release by AtT20 cells is in agreement with previous reports (24–26).

Effect of glucocorticoid pre-treatment

In order to evaluate whether pre-treatment of the cells with glucocorticoids influenced the sensitivity to the effects of OCT and SOM230, mouse AtT20 cells were pre-treated with 10 nmol/l DEX during 48 h, after which a 3 h incubation without or with CRH (10 nmol/l), OCT (1 nmol/l) and/or SOM230 (1 nmol/l) was performed. Figure 7 shows that pre-treatment of AtT20 cells with 10 nmol/l DEX had no effect on the inhibitory effect of SOM230 on CRH-induced ACTH release. On the other hand, the inhibitory effect of 1 nmol/l OCT was completely abolished by DEX pre-treatment. In the presence of DEX, SOM230 almost completely abolished CRH-induced ACTH release (Fig. 7, lower panel). Finally, DEX pre-treatment did not change the inhibitory effects of 10 nmol/l OCT or SOM230 on CRH-induced ACTH release (data not shown), suggesting that OCT at 10 nmol/l induced its inhibitory effect via interaction with sst_5, for which it has significantly lower affinity
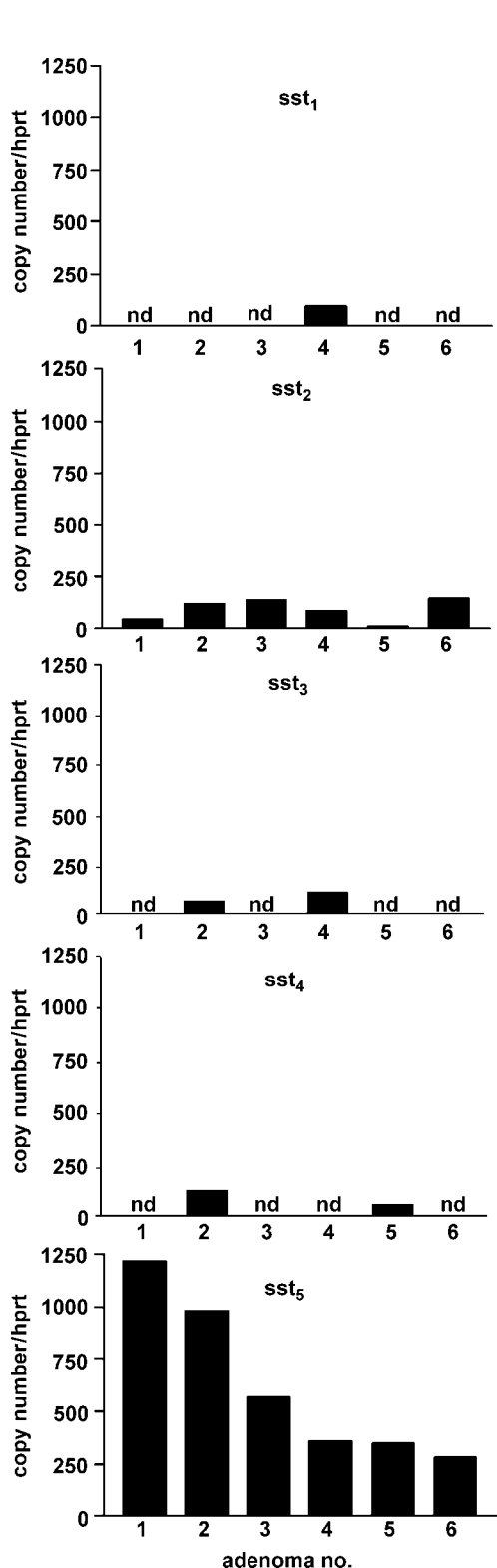
Figure 1 sst mRNA expression level in human corticotroph adenomas. mRNA levels were determined by real-time PCR of cDNA obtained from six human corticotroph adenomas (nos 1–6). Values are expressed as the number of copies relative to HPRT mRNA.
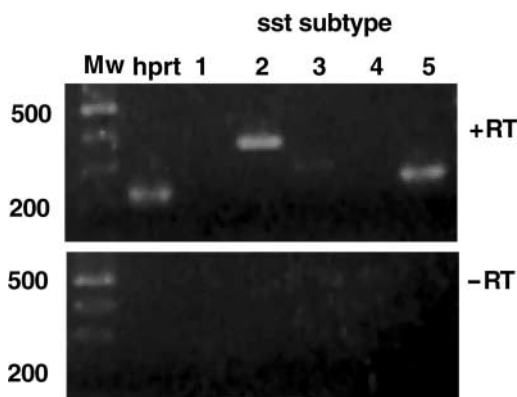

Figure 2 Expression of sst subtypes in mouse AtT20 pituitary tumour cells. Poly A^+ mRNA was reverse transcribed and cDNA was amplified by PCR. PCR products of the sst_{1-5} were separated on 1% agarose gel and stained with ethidium bromide. Upper panel represents cDNA synthesis in the presence of reverse transcriptase (+RT); lower panel represents negative control of cDNA synthesis in the absence of RT (−RT) to exclude the presence of genomic DNA contamination. Only bands of sst_2, sst_3 and sst_5 PCR products with the expected molecular weight (Mw) were detected.

compared with SOM230 (Table 1). Moreover, in one primary culture of corticotroph adenoma cells (case no. 4), which was unresponsive to OCT, DEX (10 nmol/l) pre-treatment did not reduce the inhibitory effect by SOM230 on CRH (10 nmol/l)-induced ACTH release as well (−21% without DEX vs −33% in the presence of DEX).

Discussion

Currently, there is no effective medical treatment option in patients with pituitary-dependent Cushing's disease (3, 4). Therefore, in the present study we evaluated the effects of the recently developed multi-ligand stable SS analogue SOM230 (17, 18, 27), which has high binding affinity to sst_1, sst_2, sst_3 and sst_5, on ACTH release by human corticotroph tumours *in vitro*.

The role of SS in the regulation of normal ACTH release is equivocal. Previous *in vitro* studies showed no inhibitory effect of SS on basal and CRH-induced ACTH release by normal rat anterior pituitary cells (28) and perifused normal rat pituitary halves (29). On the other hand, SS inhibited CRH-stimulated ACTH release by fragments of pituitary glands (30), as well as arginine vasopressin-induced ACTH release by cultured pituitary cells from long-term adrenal-ectomized rats (31). The latter study suggests that normal corticotrophs only respond to SS in the presence of very low cortisol concentrations. Confirming this hypothesis, we demonstrated that SS inhibits CRH-stimulated ACTH secretion by normal rat anterior pituitary cells only when the cells are cultured in the absence of glucocorticoids in the culture medium (32). In addition, pre-incubation of the cells with the
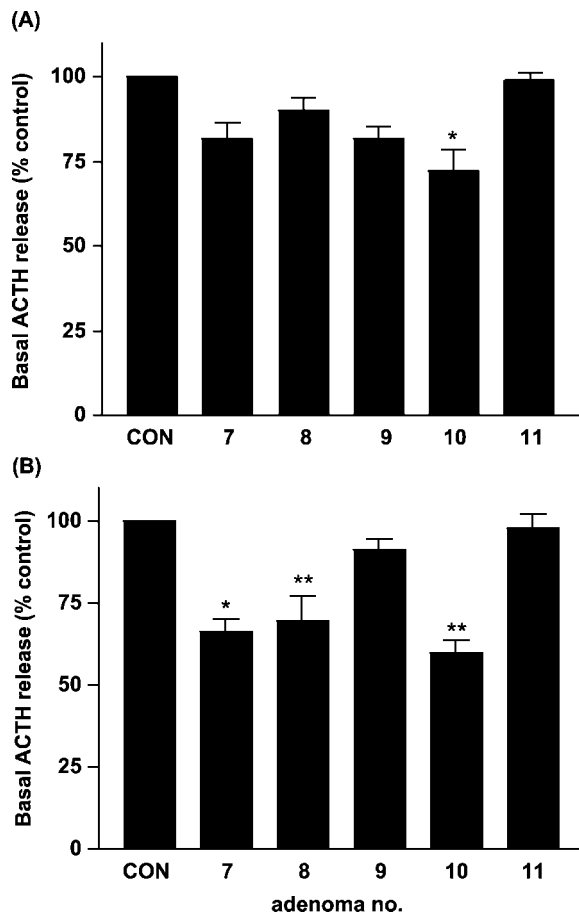
(A)

(B)

Figure 3 Effect of 10 nmol/l OCT (A) and 10 nmol/l SOM230 (B) on basal ACTH release from five cultured human corticotroph adenomas (nos 7–11). The cells were incubated in the absence or presence of OCT (A) or SOM230 (B) during 72 h after which the medium was collected for ACTH determination. Values are expressed as per cent of control (CON; untreated cells). Basal ACTH concentrations in the culture media were 239 ± 5.0, $28\,800 \pm 1159$, $25\,100 \pm 2150$, 218 ± 12 and 3228 ± 153 fmol/well, for cultured adenomas nos 7, 8, 9, 10 and 11 respectively. $*P < 0.05$ and $**P < 0.01$ vs control. Means $\pm$ S.E., $n = 4$ wells per treatment group.

progesterone-glucocorticoid receptor-blocking compound RU 38 486, increased the sensitivity of ACTH secretion to the inhibitory effect by SS and pre-treatment with DEX made the cells insensitive to SS (32). The high levels of cortisol in patients with pituitary-dependent Cushing's disease can thus be responsible for the observed lack of inhibition of ACTH release by SS and/or OCT in these patients (6, 11, 33). Moreover, in patients with Nelson syndrome and adrenal insufficiency of different origins, SS and/or OCT lower ACTH secretion (6, 9, 34, 35). In cultured corticotroph adenomas, SS and/or OCT inhibit ACTH secretion in part of the cultures (11, 36–38). Stalla *et al.* (11) showed that hydrocortisone treatment *in vitro* abolished the inhibitory effect of OCT on ACTH secretion, possibly due to a downregulation of sst on the corticotrophs.

In agreement with this hypothesis, cortisol reduced $[^{125}\text{I-Tyr}^{1}]\text{SS}$ binding on mouse AtT20/D16 pituitary tumour cells by 20–40% (39), although the sst subtype was not characterized.

Little is known with respect to sst subtype expression in pituitary corticotrophs. In rat pituitary cells, co-localization of all five sst subtypes with ACTH-expressing cells has been reported (40). In another study, sst_5 mRNA was found in 38% of normal corticotrophs, and sst_2 in only 3%. Moreover, the average number of grains per cell was also higher for sst_5 than sst_2 (12). This suggests that under normal physiological conditions, sst_5 is predominantly more expressed in rat corticotrophs than sst_2. As far as is known, no data on the quantitative expression of sst mRNA in human corticotroph adenomas are available. In the present study we found that at the mRNA level, sst_5 was the predominantly expressed receptor type in human corticotroph adenomas. In the small series of tissues ($n = 6$) analysed in this study, two cases of hyperplasia of corticotroph cells expressed a relatively high sst_5 mRNA level. On the other hand, one corticotroph adenoma expressed the highest sst_5 mRNA level in this series. The number of cases is too low, however, to establish whether there is a difference in sst_5 mRNA expression between hyperplasia of ACTH-producing cells and ACTH-secreting adenoma. sst_2 mRNA was expressed in all cases as well, although at a much lower level. The high frequency of expression of sst_2 and sst_5 in corticotroph adenomas is well in agreement with other reports. mRNA expression in human corticotroph tumours shows the overall presence of sst_1 in five out of eight (63%), sst_2 in seven out of eight (88%), sst_3 in three out of nine (33%), sst_4 in one out of eight (12%), and sst_5 in six out of seven (86%) cases (13–15, 41, 42). The low expression levels of sst_2 may explain the lack of efficacy of the sst_2-preferring agonist OCT in lowering circulating ACTH and cortisol levels in untreated patients with pituitary-dependent Cushing's disease (6, 11). *In vitro* we found a statistically significant inhibition of basal ACTH release by OCT in only one out of five cases. The higher number of cultures (three out of five) responding to a maximally active concentration of 10 nmol/l SOM230, which has very high binding affinity for sst_5, is in agreement with the higher expression levels of sst_5 mRNA. Unfortunately, not enough tissue was obtained to perform both mRNA analysis and cell culture studies. In one adenoma, which had an intermediate sst_5 mRNA level (case no. 4), SOM230 already inhibited ACTH secretion, whereas OCT had no effect. It should be noted that only mRNA levels were studied. Future studies should demonstrate whether sst_5 receptors are expressed in corticotroph adenomas at a high protein level as well. The importance of sst_5 in regulating ACTH release was further confirmed by our observation that SOM230 was also much more potent compared with OCT in inhibiting basal ACTH release by mouse AtT20/D16V corticotroph tumour cells. The pattern of inhibition, e.g. a higher potency of SOM230 vs OCT, is
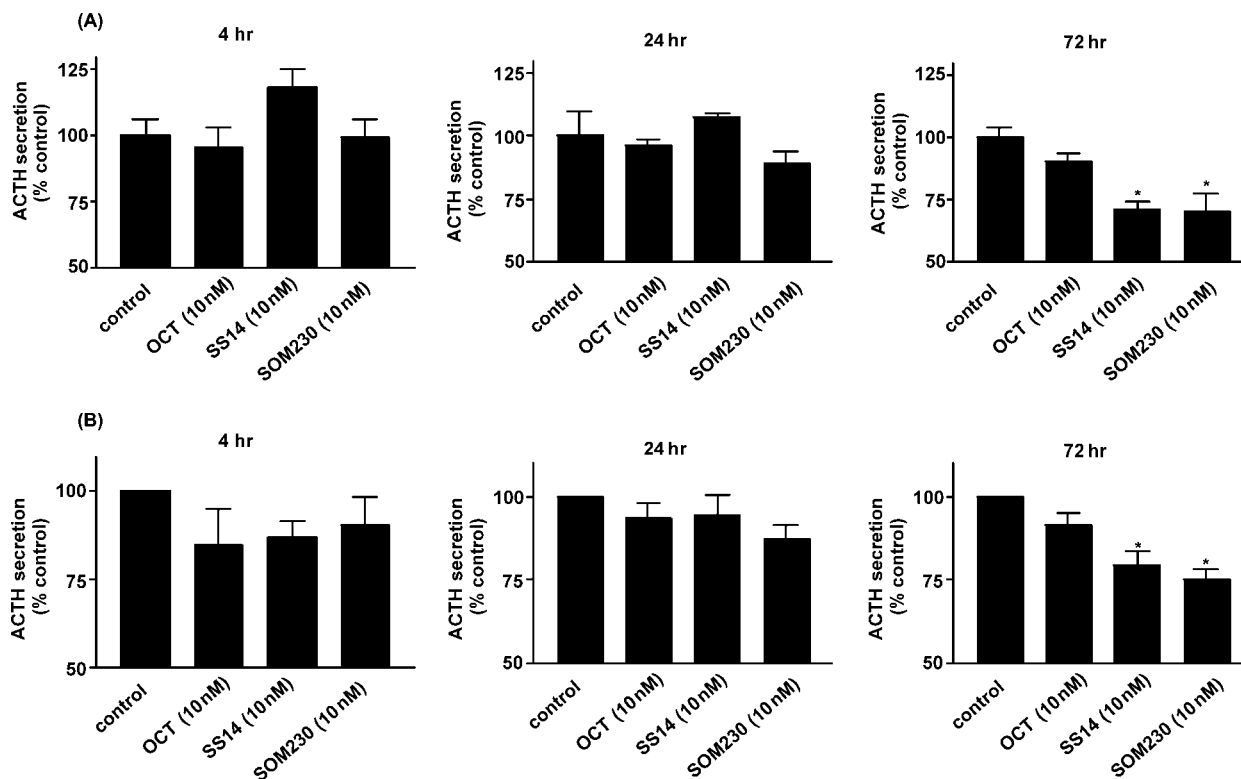
Figure 4 Time-dependent effect of OCT (10 nmol/l), SOM230 (10 nmol/l) and SS-14 (10 nmol/l) on basal ACTH release from cultured human corticotroph adenoma no. 8 (A) and mouse AtT20 corticotroph tumour (B) cells. The cells were incubated for 4, 24 or 72 h in the absence or presence of the indicated compounds, after which medium was collected for ACTH determination. Values are expressed as the per cent of ACTH release by control (untreated) cells at the indicated time-points. For the human corticotroph adenoma cells, basal ACTH values were 3505 ± 232 (4 h), 9778 ± 946 (24 h) and $28\,800\pm1159$ (72 h) fmol/well. *$P < 0.01$ vs control. Means$\pm$S.E., $n = 4$ wells per treatment group.

in line with the sst binding profile of both SS analogues. Recently, Cervia *et al.* (43) showed that sst_2 is the predominant functional receptor subtype in AtT20 cells, while sst_5 is also able to mediate inhibition of ACTH release when the ligand is not able to activate sst_2 receptors. Interestingly, while SOM230 was much more potent in inhibiting basal ACTH release by AtT20 cells, we found that OCT also potently inhibited CRH-induced ACTH release. However, maximal inhibition of ACTH by SOM230 was significantly higher compared with OCT. These data suggest that expression of sst_2 in our AtT20 line is relatively low, compared with sst_5. The involvement of both sst_2 and sst_5 in the regulation of ACTH release is further underlined by the observation that sst_2- and sst_5-specific agonists potently inhibit ACTH release and cAMP production by AtT20 cells (44).

As indicated above, glucocorticoids may downregulate sst on AtT20 corticotrophs and lower responsiveness of ACTH to the inhibitory effect of SS and/or OCT. Therefore, we also studied whether pre-treatment of the cells with the synthetic glucocorticoid DEX (10 nmol/l) reduced responsiveness of the cells to inhibition of ACTH release by SOM230. We found that glucocorticoid pre-treatment did not influence the

inhibitory effect of SOM230 on CRH-induced ACTH release, suggesting that the expression of functional sst_5 receptors is relatively resistant to glucocorticoids. This may also explain the higher expression levels of sst_5 mRNA we found in human corticotroph adenomas from untreated patients with pituitary-dependent Cushing's disease. These data suggest that glucocorticoids may have differential regulatory effects on sst_2 and sst_5 expression. An intriguing observation is that SOM230 and SS-14 only inhibit basal ACTH secretion after prolonged *in vitro* exposure. SOM230 did not inhibit AtT20 cell proliferation. Therefore, other mechanisms, e.g. inhibition of pro-opiomelanocortin synthesis and/or increased ACTH breakdown, as has been shown for the effect of OCT on GH-secreting pituitary adenomas (45, 46), may form additional explanations for the inhibitory effect of SOM230 and SS-14 on basal ACTH secretion. Interestingly, recent evidence suggests that sst_5 is also important in the control of ACTH secretion *in vivo*. sst_5 knock-out mice were shown to have significantly elevated ACTH and corticosterone levels, compared with wild-type mice (47).

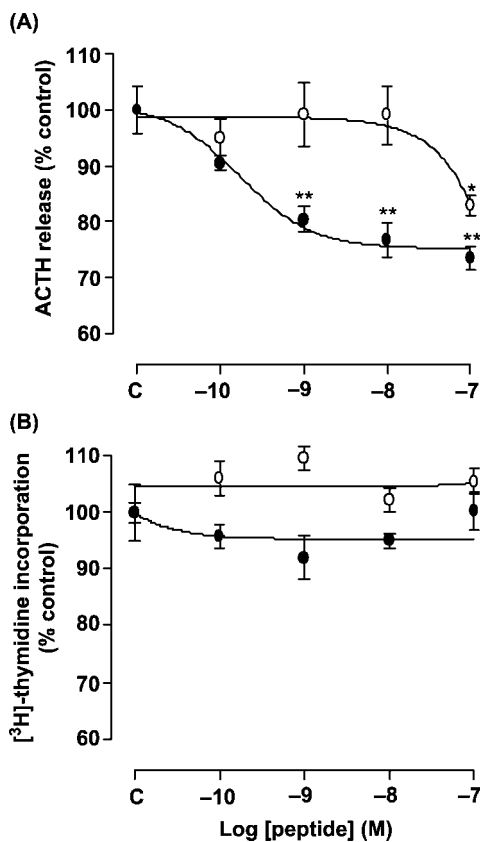While our data suggest that the novel SS analogue SOM230 may be useful in the medical management of

Figure 5 Dose-dependent effect of SOM230 and OCT on basal ACTH release (A) and cell proliferation as measured by [^{3}H]thymidine incorporation (B) by mouse AtT20 pituitary tumour cells. AtT20 cells were incubated during 72 h without or with increasing concentrations of OCT (○) or SOM230 (●), after which the medium was collected for ACTH determination. Values are expressed as the per cent of control (untreated) cells. *$P < 0.05$ and **$P < 0.01$ vs control. Means±s.e., $n = 4$ wells per treatment group.

patients with pituitary-dependent Cushing's disease, several issues will have to be clarified. First, prolonged treatment of AtT20 cells with SS-14 results in desensitization of its inhibitory effect on ACTH secretion and cAMP formation (48, 49). In addition, prolonged agonist exposure of AtT20 cells with SS-14 or SS-28 was shown to downregulate SS-14 receptors (50). However, these studies only evaluated the acute inhibitory effects of SS-14 on AtT20 cells. Moreover, the effects of SOM230 are most likely sst$_5$-mediated and sst$_5$ receptors have shown to be rapidly recycled and recruited from intracellular storages after agonist activation. This combination of recycling and recruitment of spare sst$_5$ receptors may protect from long-term downregulation through sequestration and, therefore, facilitate extended SS signalling (51). We observed that the inhibitory effects of SOM230 on basal ACTH secretion became only apparent after prolonged exposure. This observation already suggests that endogenously expressed sst$_5$ receptors may be more resistant to desensitization and/or downregulation. Nevertheless, the *in vivo* effect
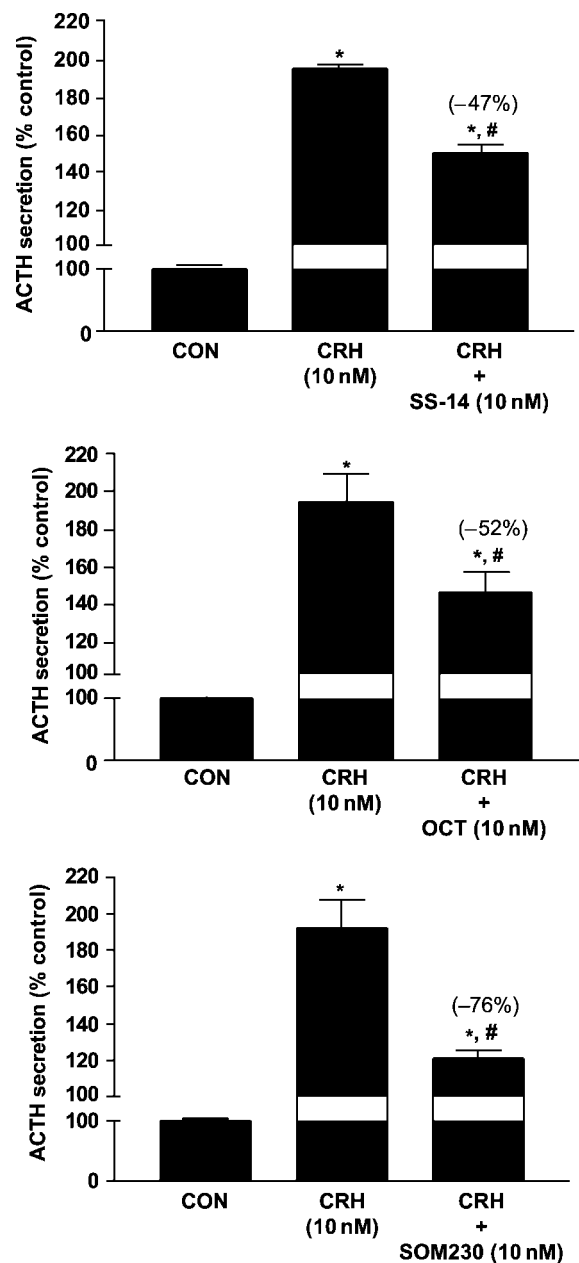

Figure 6 Effect of OCT, SS-14 and SOM230 on CRH-stimulated ACTH release by mouse AtT20 pituitary tumour cells. AtT20 cells were incubated for 3 h in the absence or presence of CRH together with either SS-14, OCT or SOM230, after which the medium was collected for ACTH determination. Values are expressed as the per cent of control (untreated) cells. *$P < 0.01$ vs control, [#]$P < 0.01$ vs CRH alone. Means±s.e., $n = 4$ wells per treatment group.

of SOM230 on ACTH secretion by corticotroph adenomas needs to be further evaluated.

In conclusion, the selective expression of sst$_5$ receptors in human corticotroph adenomas, in combination with the inhibitory effect of SOM230 on basal and CRH-induced ACTH secretion, suggests that SOM230 may have potential in the medical treatment of pituitary-dependent Cushing's disease.
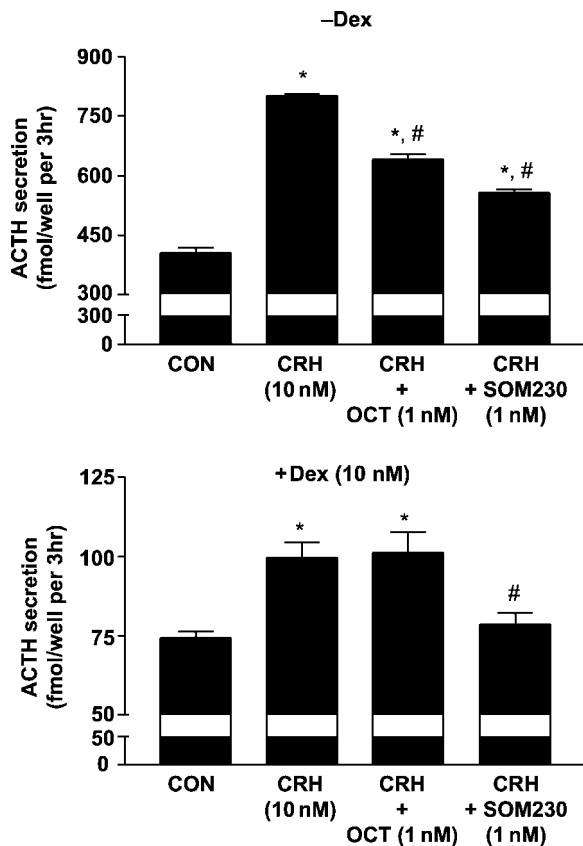
Figure 7 Effect of glucocorticoids on OCT- and SOM230-mediated inhibition of CRH-stimulated ACTH release by mouse AtT20 pituitary adenoma cells. AtT20 cells were pre-incubated during 48 h without or with 10 nmol/l DEX. After 48 h, the medium was refreshed and the cells were incubated for 3 h in the absence or presence of DEX, CRH (10 nmol/l) and OCT (1 nmol/l) or SOM230 (1 nmol/l), after which the medium was collected for ACTH determination. $*P < 0.01$ vs control, $\#P < 0.01$ vs CRH alone. Means$\pm$S.E., $n = 4$ wells per treatment group.

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