

# Differential Regulation of Somatostatin Receptor Type 2 (sst 2) Expression in AR4–2J Tumor Cells Implanted into Mice during Octreotide Treatment<sup>1</sup>

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## ABSTRACT

Octreotide is a somatostatin analogue that is widely used for cancer therapy and tumor imaging. Its efficacy in tumors depends mainly on the expression of the somatostatin receptor type 2 (sst 2). Desensitization and down-regulation of sst 2 after agonist exposure can have important consequences for patients under ongoing octreotide therapy because it may induce temporary tumor unresponsiveness and impair sst 2-based tumor scintigraphy. Therefore, we have investigated the effect of octreotide on sst 2 expression *in vitro*, as well as in a tumor mouse model.

*In vitro*, short exposure to octreotide induced rapid dose-dependent down-regulation of sst 2 in the rat pancreatic AR4–2J cell line. Within 0.5 h, 80% of sst 2 had disappeared from the cell surface. A total recovery required 24 h and was shown to depend on protein synthesis, but not on new sst 2 mRNA transcription, indicating that sst 2 was probably degraded during the down-regulation process. Similar results were obtained *in vivo*. On the other hand, long-term continuous release of octreotide for 7 days, as achieved with octreotide-containing osmotic minipumps, caused sst 2 up-regulation *in vivo*, but not *in vitro*. Furthermore, this up-regulation of sst 2 in tumor-bearing scid mice was shown to depend on constant exposure of the animals to octreotide, as it was not observed when octreotide was given discontinuously in two s.c. daily injections. These results demonstrate that the continuous release of a small amount of octreotide, which in cancer therapy may be achieved with long-acting release formulations of the peptide, can induce sst 2 up-regulation on cancer cells. This may improve the efficacy of both tumor imaging and long-term octreotide therapy.

## INTRODUCTION

The regulation of GPCRs<sup>3</sup> plays a key role in the control of hormonal responses. Exposure of GPCRs to their agonists frequently induces a rapid decrease of the initial response. This process, commonly referred to as desensitization, has been shown to involve several mechanisms such as receptor uncoupling, internalization and degradation of receptors, and decrease in receptor synthesis. For many GPCRs, the desensitization process is associated with receptor down-regulation (1–4). The five sst subtypes identified, to date (5), belong to the GPCR family. The processes of desensitization/resensitization after agonist interaction are of considerable clinical interest because long-acting analogues of somatostatin, such as octreotide, are widely used for the treatment of various endocrine tumors and acromegaly (6–9), as well as for the tumor localization by sst scintigraphy (8, 10–13).

There are conflicting *in vitro* data regarding the desensitization of the different sst subtypes after exposure to agonists. Several studies indicate that somatostatin is internalized in pancreatic acini (14), islet cells (15), pituitary cells (16), and neuroblastoma cells (17). In con-

trast, Presky and Schonbrunn (18) showed that exposure of GH4C1 pituitary cells to somatostatin does not cause desensitization and rapid internalization of sst, but instead leads to up-regulation of sst on plasma membranes. Similarly, Sullivan and Schonbrunn (19) reported that [<sup>125</sup>I][Tyr<sup>11</sup>]-somatostatin was not internalized in RINm5F insulinoma cells. These discrepancies may be interpreted by a differential capacity of internalization of the different sst subtypes and by differences in sst subtype expression in the different cell lines. This hypothesis was confirmed by Hukovic *et al.* (20), who demonstrated maximum internalization for sst 3, followed by sst 5, sst 4, and sst 2 and virtually no internalization for sst 1 in CHO-K1 cells transfected with the five sst subtypes. Nevertheless, it is very likely that the desensitization/resensitization process of sst is much more complex, and multiple mechanisms contribute to the homologous regulation of sst expression. In support of this, somatostatin has been shown to regulate sst mRNA expression in GH3 cells (21).

*In vivo* sst regulation is even more complex because somatostatin and somatostatin analogues control the release of various hormones and growth factors, which in turn can regulate the expression of sst. Moreover the phenomenon of desensitization/resensitization of GPCRs is well documented *in vitro*, but whether it occurs *in vivo* is unclear. Some indirect clinical observations indicate that sst desensitization/down-regulation does not take place in patients after a continuous octreotide treatment, but this remains largely controversial (22–24).

The goal of our study was to compare the effect of octreotide on sst expression in the same type of cancer cells *in vitro* and in a tumor mouse model. The well-characterized AR4–2J rat pancreatic cell line (25) was used because of its exclusive constitutive expression of one of the three octreotide receptors, the sst 2 subtype, that was shown to be critical for the efficacy of octreotide in the localization and treatment of tumors (26, 27). We report that a single application of octreotide induces rapid down-regulation of sst 2 both *in vitro* and *in vivo*. The recovery of normal levels of sst 2 is complete within 24 h and, in contrast to many other GPCRs, requires new receptor synthesis. Interestingly, long-term continuous exposure to octreotide *in vivo* does not cause long-lasting down-regulation of sst 2, as expected from the *in vitro* data, but leads to up-regulation of these receptors. We discuss these findings regarding their possible diagnostic and therapeutic consequences.

## MATERIALS AND METHODS

### Materials

Actinomycin D, cycloheximide, and enzyme inhibitors such as bacitracin, soybean trypsin inhibitor, phenyl methyl sulfonyl fluoride, and 1,10-phenanthroline were purchased from Sigma Chemical Co. (St. Louis, MO) and were of analytical grade. Culture media and supplements were all obtained from Life Technologies, Inc. (Basel, Switzerland). Octreotide was obtained from Novartis Pharma Inc. (Basel, Switzerland).

### Radioligand

[Tyr<sup>3</sup>]-octreotide was iodinated using the Enzymobead reagent (Bio-Rad Laboratories, Richmond, CA) consisting of immobilized lactoperoxidase and

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<sup>3</sup> The abbreviations used are: GPCR, G protein-coupled receptor; RT-PCR, reverse transcription PCR; sst, somatostatin receptor.

glucose oxidase. [ $^{125}\text{I}$ ]-octreotide [18  $\mu\text{g}$  dissolved at 2  $\mu\text{g}/\mu\text{l}$  in 2% acetic acid and further diluted with 50  $\mu\text{l}$  of 0.3 M sodium phosphate buffer (pH 7.2)] was mixed with 1 mCi  $\text{Na}^{125}\text{I}$  (NEN Life Science Products, Boston, MA), followed by the addition of Enzymobeads, suspended in 50  $\mu\text{l}$  of  $\text{H}_2\text{O}$ , and 40  $\mu\text{l}$  of 1%  $\beta$ -D-glucose. After incubation at room temperature for 1 h, the reaction was stopped by the addition of 20  $\mu\text{l}$  of saturated ascorbic acid, followed by 500  $\mu\text{l}$  of 0.25% BSA. The beads were removed by centrifugation, and [ $^{125}\text{I}$ ]-octreotide was purified in two steps: (a) on a reversed-phase mini-column filled with Spherisorb ODS 10  $\mu\text{m}$  of RP-silica (Phase Separation Inc., Norwalk, CT); and (b) by reversed-phase high-performance liquid chromatography.

### *In Vitro* Regulation of sst 2 Expression

**Time Course and Dose-dependent Regulation.** AR4-2J cells (American Type Culture Collection, Manassas, VA), previously expanded in DMEM containing 10% FCS, 2 mM L-glutamine, 50 units/ml penicillin, and 50  $\mu\text{g}/\text{ml}$  streptomycin, were stimulated for the indicated period of time with octreotide (100 nM, unless otherwise specified). The cells were detached with trypsin/EDTA (0.05/0.02%, w/v) for 3 min at 37°C. After one wash, the cell pellet was resuspended in 2 ml of cold acid buffer [40 mM sodium acetate (pH 4.5) containing 0.9% NaCl and 10% FCS] for 2 min to remove receptor-bound octreotide. The cells were then washed once and resuspended in binding medium (RPMI 1640 containing 25 mM Hepes, 0.2% BSA, and 1 mM 1,10-phenanthroline). Cells ( $5 \times 10^6$ ) were incubated for 2 h at 22°C with 200,000 cpm radioligand in the absence (total binding) or presence (nonspecific binding) of  $10^{-6}$  M octreotide. Cell-bound radioactivity was separated from free by centrifugation through a silicon oil layer, and the bound fraction was counted in a Packard  $\gamma$ -counter. Results are expressed as the percentage of specific binding (total binding minus nonspecific binding) obtained for unstimulated cells. For long-term stimulation of cells (3, 7, or 14 days), Alzet mini-osmotic pumps (model 1002; Alza, Palo Alto, CA) filled with 100  $\mu\text{l}$  of octreotide (140 or 420  $\mu\text{g}/\text{ml}$  in 0.9% NaCl) were added to culture flasks containing AR4-2J cells in 70 ml of culture medium. Under these conditions, 1 or 3  $\mu\text{g}/\text{day}$  of octreotide were released into the medium, corresponding to 0.5 or 1.5  $\mu\text{g}/\text{day}$  of octreotide for a mouse with an estimated volume of 35 ml. After 3, 7, and 14 days, one part of the cells was used for a binding experiment, as described above, and the other part was transferred to a new culture flask, together with the minipumps. The medium in this flask consisted of 25 ml of medium of the previous passage and 45 ml of fresh medium containing 1  $\mu\text{g}$  or 3  $\mu\text{g}$  of octreotide, respectively.

**Reappearance of sst 2 after the Down-Regulation.** AR4-2J cells were stimulated with 100 nM octreotide for 16 h to induce the down-regulation of sst 2. Octreotide was then removed by several washes, and fresh medium containing either 100  $\mu\text{M}$  cycloheximide or 4  $\mu\text{M}$  actinomycin D (or solvent for controls) was added to the cells. After different times of incubation, the number of sst 2 was evaluated by a binding experiment, as described above.

### *In Vivo* Regulation of sst 2 Expression

CB17 scid mice (breeding pairs obtained from IFFA-CREDO, L'Arbresles, France) were implanted s.c. with  $1 \times 10^6$  AR4-2J cells. After 1 week, when the tumors reached about 1 cm in diameter, octreotide or NaCl (controls) was injected i.v. in 200- $\mu\text{l}$  quantities, as indicated. For continuous release of octreotide, Alzet mini-osmotic pumps filled with 100  $\mu\text{l}$  of octreotide (70  $\mu\text{g}/\text{ml}$  in NaCl) were implanted s.c. in the rear flank of the animals when the tumors became visible. This results in continuous exposure of the mice to 0.5  $\mu\text{g}$  of octreotide/day. After various periods of time, the tumors were excised and immediately frozen at  $-80^\circ\text{C}$ . For long-term discontinuous administration, 0.25  $\mu\text{g}$  of octreotide was administered s.c. twice daily in 100  $\mu\text{l}$  (9 h and 17 h). Determination of sst 2 expression was performed as described previously (28). Briefly, tumor samples were homogenized with a Polytron homogenizer in ice-cold 20 mM Tris buffer (pH 7.5) containing 0.25 M sucrose, 1 mg/ml bacitracin, 0.1 mg/ml soybean trypsin inhibitor, and 0.125 mg/ml phenyl methyl sulfonyl fluoride (buffer A), plus 1 mM EDTA. The lysates were centrifuged at  $500 \times g$  at 4°C for 5 min to remove nuclei and cell debris, and the supernatants were centrifuged at  $40,000 \times g$  at 4°C for 50 min after a 5-min incubation with cold acid buffer (sodium acetate; final pH 4.5, after mixing with the supernatant). The pelleted membranes were resuspended in 500  $\mu\text{l}$  of buffer A, and the protein content was determined by the method of Bradford

(Bio-Rad, Hercules, CA) using BSA as standard. Membrane preparations were kept at  $-80^\circ\text{C}$  until use. Determination of the number of sst 2 was performed by binding experiments. Triplicates of 100- $\mu\text{l}$  membranes adjusted to the appropriate concentration (usually 0.5 mg/ml) in buffer A were incubated with either 50  $\mu\text{l}$   $4 \times 10^{-6}$  M octreotide (determination of nonspecific binding) or 50  $\mu\text{l}$  of medium (determination of total binding) and 50  $\mu\text{l}$  of radioligand (50,000 cpm) in 96-well U-bottomed microplates (Falcon 3077; Becton Dickinson, Franklin Lakes, NJ) at 37°C for 1 h. Both octreotide and radioligand were diluted in binding medium, which consisted of modified Eagle's medium supplemented with 25 mM Hepes, 0.4% BSA, 1 mM 1,10-phenanthroline, and a mixture of protease inhibitors (Sigma P8340; 0.05  $\mu\text{l}/\text{well}$ ). The reaction was stopped by placing the microplates on ice for 10 min, and the membrane-bound radioactivity was collected on filters with the help of a cell harvester (Packard, Meriden, CT). The radioactivity was counted in a microplate scintillation counter (TopCount; Packard). Specific binding was calculated by subtracting the nonspecifically bound radioactivity from that of the total binding.

### Quantitative Determination of sst 2 mRNA

Total RNA was extracted from tumors in 10 volumes of TriZOL reagent (Life Technologies, Inc.), according to the manufacturer's protocol. Total RNA (30  $\mu\text{g}$ ) was subjected to a DNase I (Boehringer Mannheim, Rotkreuz, Switzerland) digest, followed by a clean-up with the RNeasy kit (QIAGEN, Basel, Switzerland). First-strand cDNA was produced by Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.), using 1  $\mu\text{g}$  of total RNA and 200 ng of oligo(dT)15. For each RNA preparation, genomic DNA removal was controlled by omitting reverse transcriptase. The first-strand cDNA was then quantified by using the TaqMan fluorescence-based PCR assay according to the manufacturer's protocol (Perkin-Elmer Corp., Foster City, CA). In practice, cDNA corresponding to 25 ng of RNA was mixed in quadruplates with a master mixture that contained all reagents for PCR and supplemented with either sst 2- or actin-specific probe (225 nM) and primers (900 nM; sst 2 primers: 5'-GAGGACACGATGGCCTGG-3', 5'-CACGCGCGGAACCTTGA-3'; actin primers: 5'-GCTGTCACCTTCCCGGTGT-3', 5'-CACTCCAAGTATCCACGGCATA-3'; sst 2 probe: 5'-CCCGTGGAAGCA GCTACCCG-3'; actin probe: 5'-ACTCAGGGCATGGA-TGCAGCCATC-3') in a final volume of 25  $\mu\text{l}$ . The primers and probes were designed and proved to be specific for rat without cross-reactivities with mouse to ensure that the RNA detected was of tumor- and not host-origin. The probes were labeled with the fluorescent dyes 5-carboxyfluorescein (FAM) on the 5' end and N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3' end. Amplification and detection were performed with the ABI 7700 system with the following profile: 1 cycle of 50°C for 2 min, 1 cycle or 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. In these conditions, the efficiencies for target (sst 2) and reference (actin) were found to be equal, therefore, it was not necessary to include a standard curve on each plate. Data were analyzed as described by the manufacturer by comparison of the threshold cycles (CT, fractional cycle number at which the amount of amplified target reaches a fixed threshold) normalized to actin. Results are expressed relative to the control mean value (set as 1).

### sst 3 and sst 5 mRNA Detection

The detection of sst 3 and sst 5 mRNA was performed by classical PCR after generation of cDNA, as described above. The sense and antisense primers for sst 3 were 5'-TCTCGGCGAGTACGGAGCCA-3' and 5'-ACAGATG-GTCCAGCGTGCTG-3', respectively, and the sense and antisense primers for sst 5 were 5'-TGCTCCAGTGGTAACCATA-3' and 5'-AATAATACGT-CAGCCACGGC-3', respectively. Annealing temperatures were 62°C for sst 3 and 58°C for sst 5, and either 35 or 60 cycles were performed. As a positive control, 100 ng of rat genomic DNA (Clontech Laboratories, Palo Alto, CA) were used because ssts are intronless.

### Analysis of Data

Results were expressed as the mean  $\pm$  SE. Statistical analysis of the data were performed using a one-way ANOVA test. When significant overall effects were obtained by ANOVA, multiple comparisons were made with the Bonferroni correction. For comparisons of one-site with two-site competitive binding curves, an F test was used. A  $P < 0.05$  was considered to indicate a statistically significant difference.

## RESULTS

**Expression of sst 2, sst 3, and sst 5 by AR4–2J Cells before and after Passage *in Vivo*.** Octreotide binds to sst 2, sst 5, and, to a lesser extent, sst 3. The subtype(s) expressed in AR4–2J cells before and after passage in scid mice was analyzed by RT-PCR. As illustrated in Fig. 1, only sst 2 mRNA was detected in these cells, even after passage *in vivo*. No specific bands corresponding to the expected sizes of sst 3 and sst 5 cDNA were visible. This result was confirmed by control experiments using up to 60 PCR amplification cycles. Thus, AR4–2J cells can be considered as cells expressing, exclusively, sst 2.

**Validation of the Method to Remove Surface-bound Octreotide.**

To determine the number of cell surface sst 2 by binding experiments after octreotide pretreatment of the cells, it was necessary to develop a procedure for maximal dissociation of the octreotide bound to sst 2. The acid wash procedure described in “Materials and Methods” is the result of a series of experiments (data not shown) in which the conditions were optimized to achieve both maximal cell viability (>80%) and maximal dissociation of octreotide from the receptor (85–90%), as controlled with plasma membrane preparations.

***In Vitro* Down-Regulation of sst 2 Expression after Stimulation with Octreotide.** Both short-term (up to 5 h) and long-term (up to 14 days) exposure of AR4–2J cells to octreotide were performed. For short-term stimulation, octreotide was added (single addition) to AR4–2J cells at different concentrations and for various periods of time (0.5–5 h), and the level of sst 2 expression was evaluated by measuring the specific binding of [<sup>125</sup>I][Tyr<sup>3</sup>]-octreotide to the cells after removal of membrane-bound octreotide by acid wash. As shown in Fig. 2A, octreotide induced a dose-dependent down-regulation of sst 2, reaching a plateau at 100 nM. At this point, the specific binding was 20–30% of that observed with untreated cells, suggesting that 70–80% of sst 2 were down-regulated. This sst 2 down-regulation was rapid (0.5 h) and stable over time (at least 5 h; Fig. 2B). Long-term exposure of AR4–2J cells to octreotide, as performed with the help of minipumps containing octreotide, indicated that the down-regulation could be maintained for at least 14 days (Fig. 2C). Thus, octreotide induced a rapid down-regulation of sst 2 *in vitro*, which persisted for at least 14 days.

***In Vitro* Reappearance of sst 2 on the Cell Surface after Octreotide-induced Down-Regulation.** Fig. 3 shows the kinetics of sst 2 reappearance on the cell surface after octreotide-induced down-regulation. As early as 0.5 h after octreotide removal, sst 2 reappeared and a total restoration was found within 24 h. To investigate further the mechanisms underlying the reappearance of sst 2 to the cell surface, cycloheximide or actinomycin D was added to the cells during the “reappearance time” immediately after octreotide with-

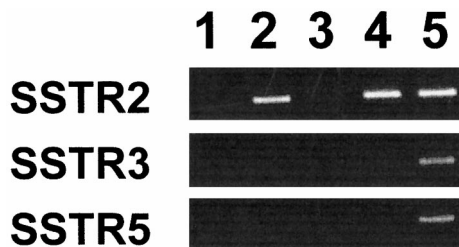


Fig. 1. RT-PCR analysis of sst 2, sst 3, and sst 5 in AR4–2J cells before and after *in vivo* passage. Photographs of the ethidium bromide-stained agarose gels of PCR products are shown. Primer pairs used for PCR were specific for sst 2 (SSTR2), sst 3 (SSTR3), and sst 5 (SSTR5). Lanes 1 and 2, AR4–2J cells cultured *in vitro*; Lanes 3 and 4, AR4–2J cells after passage *in vivo*; Lane 5, rat genomic DNA. For each RNA preparation, DNA removal was controlled by omitting reverse transcriptase (Lanes 1 and 3). The bands migrated to the expected locations for the primers used. Intactness of mRNA was controlled by using actin primers (data not shown).

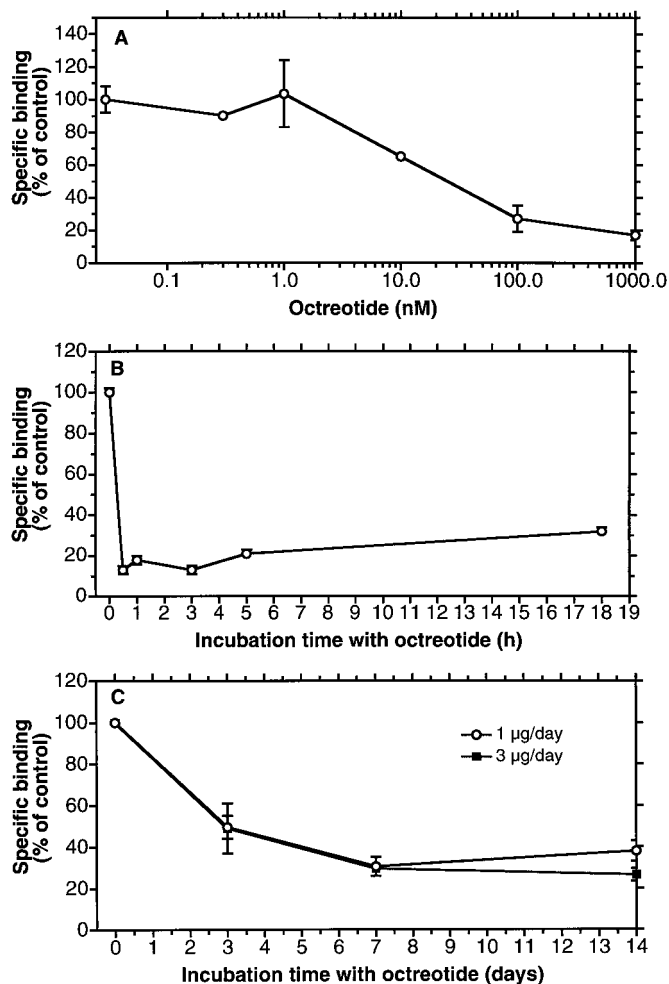


Fig. 2. Concentration dependence and time course of sst 2 down-regulation *in vitro*. AR4–2J cells were treated with octreotide, and sst 2 expression was then monitored by binding experiments using [<sup>125</sup>I][Tyr<sup>3</sup>]-octreotide. A, concentration dependence of sst 2 regulation; the duration of octreotide stimulation was of 16 h. B, time course of sst 2 regulation for short-term stimulation; a single addition of octreotide yielding a 100-nM concentration was performed. C, time course of sst 2 regulation for long-term stimulation; minipumps containing octreotide were added to the cells to ensure a continuous release of either 1 µg or 3 µg octreotide/day for the indicated periods of time. The two curves are almost superimposed. Each point represents the mean ± SE (n = 3–6).

drawal. In the presence of cycloheximide, there was a total inhibition of cell surface sst 2 restoration ( $P < 0.05$ , versus control values in the absence of cycloheximide), whereas actinomycin D did not have any significant effect ( $P > 0.05$ , versus control values in the absence of actinomycin D). This suggests that reappearance of sst 2 requires biosynthesis of a new receptor, independent of sst 2 mRNA formation.

***In Vivo* Regulation of sst 2 Expression after Single Injections of Octreotide.** Fig. 4A shows the effect of a low (1 µg) or high (50 µg) dose of octreotide administered *i.v.* at the level of sst 2 expression in AR4–2J tumors implanted into scid mice. Similar to the *in vitro* data, a single administration of octreotide led to a rapid down-regulation of sst 2, which was already maximal at the first time point considered (0.5 h). One hour after octreotide injection, the recovery of cell surface sst 2 was already initiated and was complete within 16–24 h. As depicted in Fig. 4B, this down-regulation was dose-dependent: treatment with 0.3 µg octreotide lowered specific binding to 55% as compared with controls; treatment with 50 µg lowered specific binding to a maximum of 22%. The dose of 0.3 µg/mouse corresponds to a dose of 600 µg for a 60-kg patient, which represents the dose used in the clinic for treatment of cancer or acromegaly. Thus, similar to



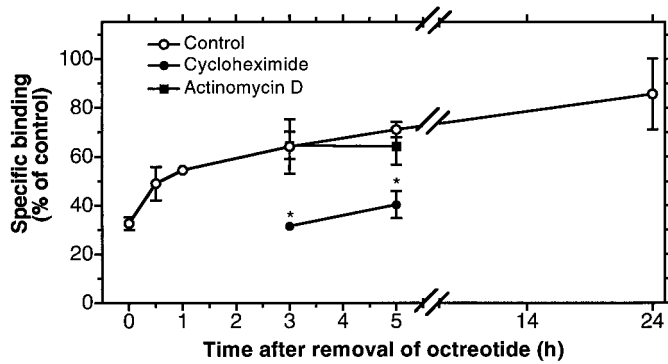


Fig. 3. *In vitro* kinetic of sst 2 reappearance after down-regulation. AR4-2J cells were pretreated for 16 h with 100 nM octreotide. After washing off octreotide, the cells were incubated in the absence or presence of cycloheximide or actinomycin D for various periods of time. The time course of reappearance of sst 2 to the cell surface was then monitored by binding experiments using [<sup>125</sup>I][Tyr<sup>3</sup>]-octreotide. Each point represents the mean  $\pm$  SE ( $n = 3-10$ ). \*,  $P < 0.05$  versus corresponding controls.

the results *in vitro*, a single injection of octreotide induced a rapid dose-dependent down-regulation of sst 2 on AR4-2J tumors *in vivo*. This down-regulation was only transient.

***In Vivo* Regulation of sst 2 mRNA after Single Injections of Octreotide.** To determine whether octreotide regulates sst 2 mRNA in AR4-2J tumors, RNA was extracted from the tumors after various time periods after the injection of 50  $\mu$ g of octreotide and quantified. As shown in Fig. 5, octreotide induced only a minor (1.3 x) and transient decrease in sst 2 mRNA in AR4-2J tumors ( $P < 0.01$  for the times 1 and 3 h versus controls), which was fully normalized within 8 h.

***In Vivo* Regulation of sst 2 Expression after Continuous Administration of Octreotide.** To imitate clinical treatment regimens using chronic octreotide administration, we have implanted octreotide-containing minipumps s.c. into tumor-bearing mice, ensuring constant release of the drug. After 3 days of continuous octreotide administration, the specific binding of [<sup>125</sup>I][Tyr<sup>3</sup>]-octreotide to AR4-2J tumors did not differ from that of untreated mice ( $P > 0.05$ ; Fig. 6), in contrast to treatment of cells *in vitro*. Unexpectedly, extension of the octreotide treatment to a period of 7 days even led to a significant increase of [<sup>125</sup>I][Tyr<sup>3</sup>]-octreotide binding (150% of control values;  $P < 0.05$ ). This elevation of specific binding was not associated with a change in receptor affinity ( $IC_{50}$  2.1  $\pm$  0.3 nmoles/liter for control versus 1.4  $\pm$  0.15 nmoles/liter for 7-day octreotide treatment;  $P > 0.05$ ). Moreover, a statistical analysis of the binding data with respect to a one-site and a two-site binding model indicated that both data sets fitted the one-site model only. Taken together, these results are compatible with an increase of sst 2 numbers as opposed to an induction of other sst subtypes, such as sst 3 or sst 5, or to a change in sst 2 affinity for octreotide.

In contrast, when octreotide was given discontinuously in two daily injections, the specific binding of [<sup>125</sup>I][Tyr<sup>3</sup>]-octreotide to AR4-2J tumors was decreased (57% of control values;  $P < 0.05$ ) 8 h after the last octreotide injection, irrespective of the duration of octreotide treatment (3 days or 7 days;  $P > 0.05$ ). Thus, *in vivo* long-term administration of octreotide induced a differential sst 2 regulation, depending on the treatment protocol: discontinuous administration of octreotide induced sst 2 down-regulation, whereas continuous exposure led to an up-regulation of sst 2.

## DISCUSSION

Octreotide plays a major role in the management of patients with acromegaly and tumors and is the primary drug for most patients who

are refractory to surgery and radiotherapy. As a radiolabeled version, octreotide is also the most often used agent for the localization of neuroendocrine tumors in nuclear medicine, and, therefore, we and others develop novel isotope-labeled octreotide analogues useful not only for tumor visualization but also for peptide receptor-mediated internal radiotherapy (12, 29-32). However, the success of octreotide in the clinic depends strictly on the expression of sst receptors and, particularly sst 2, by the tumor cells (26, 27). *In vitro* agonist-induced desensitization/down-regulation is common to many GPCRs (4), and it was also suggested for sst receptors (14-17), although it is still a matter of debate (18, 19). Despite the considerable importance of such

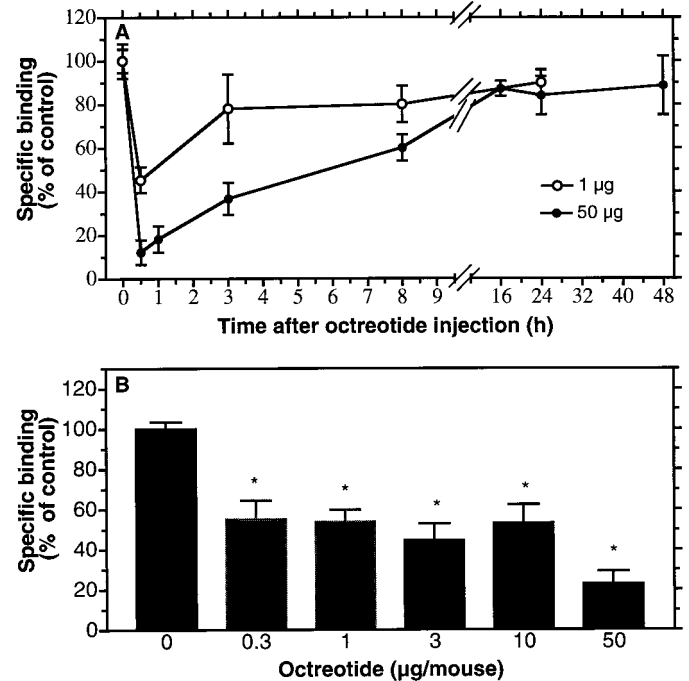


Fig. 4. Dose dependence and time course of sst 2 down-regulation *in vivo* after a single injection of octreotide. AR4-2J tumor-implanted scid mice were injected i.v. with a single dose of octreotide or NaCl as control and sst 2 expression was monitored on tumor plasma membrane preparations by binding experiments using [<sup>125</sup>I][Tyr<sup>3</sup>]-octreotide. A, time course of sst 2 regulation after application of either 1  $\mu$ g or 50  $\mu$ g octreotide. B, dose dependence of sst 2 regulation; sst 2 expression was monitored 0.5 h after octreotide injection; \*,  $P < 0.05$  versus untreated mice. Each point represents the mean  $\pm$  SE ( $n = 3-18$ ).

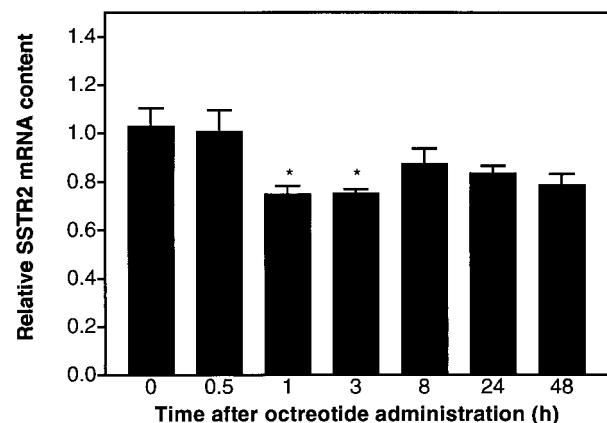


Fig. 5. *In vivo* time course of sst 2 mRNA regulation after a single injection of octreotide. AR4-2J tumor-implanted scid mice received i.v. injections of a single dose of 50  $\mu$ g of octreotide. Control mice received NaCl. After various periods of time, the mice were sacrificed and sst 2 mRNA was quantified by a quantitative PCR protocol. Results are expressed as mean  $\pm$  SE ( $n = 5$ ). \*,  $P < 0.01$  versus untreated mice.

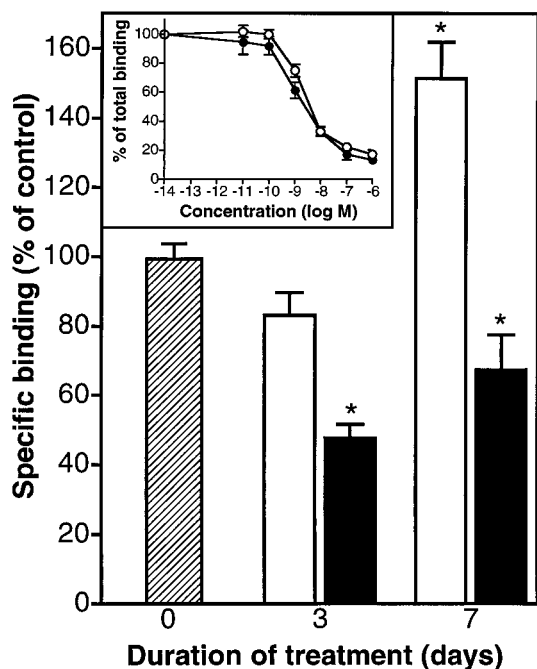


Fig. 6. *In vivo* time course of sst 2 regulation after a continuous release of octreotide. AR4-2J tumor-bearing scid mice were implanted s.c. with osmotic minipumps containing octreotide to ensure a continuous release of 0.5  $\mu\text{g}$  of octreotide/day ( $\square$ ) or received s.c. injections twice-daily of 0.25  $\mu\text{g}$  of octreotide ( $\blacksquare$ ),  $\square$ , mice treated with vehicle. After 3 or 7 days, the mice were sacrificed and sst 2 expression was monitored on plasma membrane preparations by binding experiments using [ $^{125}\text{I}$ ][Tyr $^3$ ]-octreotide. Results are expressed as mean  $\pm$  SE ( $n = 13$  for untreated mice and  $n = 5-6$  for treated mice). \*,  $P < 0.05$  versus untreated mice. Inset, competitive binding curves obtained with plasma membrane preparations from control ( $\circ$ ) and 7-day continuously treated mice ( $\bullet$ ).

a phenomenon for patients under octreotide treatment, no information has been available, to date, about octreotide-mediated regulation of sst 2 expression in cancer cells *in vivo*.

In the present study, we have addressed this question by comparing the octreotide-driven regulation of sst 2 on AR4-2J cells *in vitro* and *in vivo*, using scid mice as the animal model. An important prerequisite for these studies was the demonstration by RT-PCR that AR4-2J cells express only one of the three octreotide receptors, the sst 2. Thus, in contrast to studies performed with transfected cell lines, our approach offers the advantage of investigating sst 2 in a natural environment, including the presence of the sst 2 promoter. This can be important because the molecular mechanism and the kinetics of agonist-induced down-regulation may depend on various factors, such as the presence of additional cellular proteins or on transcription of receptor mRNA. The most intriguing finding, however, was that octreotide treatment either induced down-regulation or up-regulation of sst 2 depending on the experimental protocol. Down-regulation was observed in the following four situations: (a) short exposure to octreotide *in vitro*; (b) long exposure to octreotide *in vitro*; (c) short exposure to octreotide *in vivo*; and (d) long-term discontinuous exposure to octreotide *in vivo*. By contrast, up-regulation was found only *in vivo* after a continuous application of octreotide with osmotic minipumps. This differential homologous sst 2 regulation observed *in vivo* (and not found *in vitro*) demonstrates the difficulty to extrapolate data generated *in vitro* to the situation found *in vivo*.

The differential regulation of sst 2 expression observed after discontinuous versus continuous treatment with octreotide is also clinically relevant. In our animal model, twice daily injections of octreotide (discontinuous protocol) caused down-regulation of sst 2 at therapeutic doses, without significant change over time in the extent of down-regulation. This suggests that discontinuous administration

permits receptor regeneration between two injections, which is in accordance with clinical results showing prolonged efficacy of long-term octreotide therapy when octreotide is given two to three times daily (22-24). The use of osmotic minipumps in the animal model (continuous protocol) imitates the recently introduced "long-acting release" octreotide (LAR octreotide, bound to a polymer matrix; Ref. 23). The clinical observation of an increase in efficacy of the LAR octreotide formulation versus intermittent s.c. administration of octreotide (33) may be explained by the unexpected finding of sst 2 up-regulation in AR4-2J tumors after continuous octreotide treatment. Whether this up-regulation is associated with a concomitant enhancement of the biological response is not yet known and requires further investigations. This up-regulation of sst 2 is also of considerable interest for tumor imaging and internal radiotherapy. We are currently testing a probable improvement in tumor uptake of [ $^{111}\text{In}$ -DOTA][Tyr $^3$ ]-octreotide in our animal model, after inducing sst 2 up-regulation by a continuous release of octreotide. From a practical point of view, it is worth questioning whether the period of octreotide withdrawal before somatostatin receptor scintigraphy, as is customary in the clinical setting, is necessary for patients receiving the LAR octreotide. This may be a critical advantage in patients with severe hyperhormone secretion syndrome.

The possibility that other sst subtypes that bind octreotide, namely sst 3 and sst 5, might account for the sst 2 up-regulation can be excluded in our experimental system. First, we did not detect any sst 3 or sst 5 mRNA by RT-PCR in our AR4-2J cells not only *in vitro*, but also after passage in scid mice. Secondly, competition-binding experiments on membranes with up-regulated SSTRs revealed the presence of one class of SSTRs with an affinity similar to the one determined in control membranes. Recently, sst receptors were identified in human peritumoral blood vessels (34). Nevertheless, the hypothesis that the increase of sst binding measured in our tumor extracts originated from an enhanced binding to peritumoral veins can be excluded for different reasons. In implanted tumors, blood vessels represent only 1.5% of the tumor volume (35); thus, it is difficult to conceive that this tiny fraction would account for the marked sst 2 up-regulation. To date, sst receptors could not be identified in peritumoral veins in rodents (34).

The molecular mechanism of sst 2 down- and up-regulation is not yet clear. The *in vitro* and *in vivo* experiments dealing with short exposure of sst 2 to octreotide revealed that a single administration of octreotide to AR4-2J cells induced rapid dose-dependent down-regulation of sst 2. Approximately 80% of sst 2 disappeared from the cell surface within 0.5 h. The kinetic of sst 2 reappearance after octreotide-induced down-regulation was much slower with a  $t_{1/2}$  of 4 h and a total recovery time of 24 h, suggesting that mechanisms more complex than a simple recycling of internalized receptors were involved. This is supported by the observation that cycloheximide, a protein synthesis inhibitor, totally prevented this recovery. Thus, homologous down-regulation of sst 2 seems to be associated with receptor degradation, making *de novo* receptor synthesis necessary for the recovery process. This is in agreement with a recent confocal microscopic study postulating that sst 2 entered an endocytic pathway after agonist interaction (36). On the other hand, actinomycin D did not alter the reappearance of sst 2 after octreotide-induced down-regulation *in vitro*, indicating that the recovery of sst 2 was independent of biosynthesis of new sst 2 mRNA. Similarly, a single injection of octreotide led to a minor variation in sst 2 mRNA in tumors *in vivo*. Thus, sst 2 regulation *in vivo* after short or discontinuous exposure of AR4-2J tumors to octreotide does not differ from that found *in vitro*, indicating that sst 2 down-regulation is not indirectly influenced by the host. By contrast, sst 2 up-regulation after continuous exposure of AR4-2J tumors to octreotide was only found *in vivo*, but not *in vitro*,

which argues in favor of an indirect involvement of the host in this process. Continuous exposure to octreotide can influence the release of various hormones and growth factors *in vivo*, and some of them were shown to up-regulate *sst in vitro* (37). Nevertheless, considering the number of *in vivo* targets of octreotide, including the anterior pituitary gland and the gastroenteropancreatic endocrine system, it is difficult to speculate about the most likely indirect factor(s) responsible for this up-regulation.

In conclusion, our data show that *in vivo* the same peptide, octreotide, is capable of regulating *sst 2* expression in AR4-2J tumors in two different ways. When octreotide was applied for a short time, as is the case during a physiological hormonal stimulation with somatostatin, *sst 2* were rapidly down-regulated. By contrast, when octreotide was administered for a longer period, *sst 2* were either down-regulated (discontinuous octreotide release) or up-regulated (continuous octreotide release). To our knowledge, this is the first study demonstrating that octreotide can indirectly up-regulate its own receptor expression. These results can have important implications for tumor imaging based on *sst 2* expression and long-term efficacy of octreotide therapy. We are currently investigating whether other *sst 2* agonists used in a clinical setting, such as lanreotide or vapreotide, exhibit similar properties and whether the octreotide-mediated up-regulation *sst 2* is also found in other tumors of other species.

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