

Internalization of the Radioiodinated Somatostatin Analog [¹²⁵I-Tyr³]Octreotide by Mouse and Human Pituitary Tumor Cells: Increase by Unlabeled Octreotide*

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ABSTRACT

Recently, we developed a technique that allows the *in vivo* visualization in man of somatostatin receptor-positive neuroendocrine tumors after iv injection of [¹²⁵I-Tyr³]octreotide or [¹¹¹In-DTPA-D-Phe¹]octreotide. Radiotherapy of such tumors using somatostatin analogs coupled to α - or β -emitting radionuclides has been proposed as an application for radiolabeled somatostatin analogs. To develop this concept further, it is of importance to know whether the above-mentioned radiolabeled somatostatin analogs are internalized by the tumor cells, and whether it might be possible to manipulate the degree of internalization. In the present study we investigated the internalization of a stable somatostatin analog, [¹²⁵I-Tyr³]octreotide, by mouse AtT20/D16V pituitary tumor cells and primary cultures of human GH-secreting pituitary tumor cells. Treatment of the cells with low pH was used to distinguish between membrane-bound (acid-releasable) and internalized (acid-resistant) radioligand.

[¹²⁵I-Tyr³]octreotide showed a time-dependent increasing accumulation in AtT20 cells; after 4 h of incubation, values up to 6–8% of the dose of radioligand added were obtained. Binding and internalization of [¹²⁵I-Tyr³]octreotide were temperature dependent and inhibited by pertussis toxin. Inhibitors of lysosomal degradation did not increase the amount of internalized radioligand. After 4 h of incubation, 88% of the radioactivity present in the cells was still peptide bound, sug-

gesting a low intracellular breakdown of this radioligand. Six of seven human GH-secreting adenoma cell cultures also internalized [¹²⁵I-Tyr³]octreotide (variation between 0.24–4.98% of the dose radioligand added). Displacement of binding and internalization of [¹²⁵I-Tyr³]octreotide by unlabeled octreotide showed a bell-shaped curve in AtT20 cells. At low concentrations (0.1 and 1 nM), binding and internalization were increased, whereas at higher concentrations, saturation occurred. In contrast to this, binding of [¹²⁵I-Tyr³]octreotide to a broken cell preparation of AtT20 cells was displaced in a dose-dependent manner by unlabeled octreotide, with an IC₅₀ of 0.1 nM. Similar observations were made in the human GH-secreting adenoma cell cultures.

In conclusion, a high amount of [¹²⁵I-Tyr³]octreotide is internalized in a specific-, time-, temperature-, and pertussis toxin-sensitive GTP-binding protein-dependent manner by mouse AtT20 and human GH-secreting pituitary tumor cells. In the presence of a low concentration of unlabeled octreotide, a rapid increase in the amount of [¹²⁵I-Tyr³]octreotide internalized by AtT20 cells and by the majority of the human GH-secreting adenoma cell cultures was found. Because membrane binding was simultaneously increased, this is suggested to be related to a rapid recruitment of somatostatin receptors at the outer tumor cell membrane. (*Endocrinology* 136: 3698–3706, 1995)

SOMATOSTATIN receptors (SS-R) are present in all normal target tissues of the peptide, such as brain, anterior pituitary gland, and pancreas. In a variety of human tumors, frequently originating from normal somatostatin (SS) target tissues, high numbers of SS-R can be detected by classical biochemical binding techniques as well as by *in vitro* autoradiography. These tumors include those with amine precursor uptake and decarboxylation characteristics (pituitary tumors, endocrine pancreatic tumors, carcinoids, paragangliomas, small cell lung cancers, medullary thyroid carcinomas, and pheochromocytomas) as well as meningiomas, well differentiated brain tumors (astrocytomas), neuroblastomas, lymphomas, and some human breast cancers (1). Recently, we developed a technique that allows the *in vivo* visualiza-

tion in man of the above-mentioned SS-R-positive tumors after iv injection of [¹²⁵I-Tyr³]octreotide (2, 3) or [¹¹¹In-DTPA-D-Phe¹]octreotide (4). Using this technique, we showed that certain tumors, especially those with a high number of SS-R, could be visualized 48 h after injection (1–4). This rather long residence time of radioactivity on human tumors *in vivo* suggests that the radioligand is internalized by the tumor cells. Internalization of radioligand is of special importance when radiotherapy of certain SS-R-positive human cancers with α - or β -emitting isotopes coupled to SS analogs is considered (5, 6).

At present, equivocal data have been reported with respect to internalization of SS. Receptor-mediated endocytosis of SS has been demonstrated in rat anterior pituitary cells and rat islet cells (7–14), whereas other investigators found that [¹²⁵I-Tyr¹]SS-14 and [¹²⁵I-Tyr¹¹]SS-14 are not rapidly internalized by GH₄C₁ rat pituitary cells and RINm5F insulinoma cells, respectively, probably due to degradation of these radioligands at the cell surface (15, 16). As data with respect to internalization of SS may have been influenced by the susceptibility to degradation of the SS ligands used in the above-

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mentioned studies, investigations with respect to internalization of more stable radioligands, such as [¹²⁵I-Tyr³]octreotide (17), may provide a better insight into the amount of internalization by SS-R-positive (tumor) cells.

In the present study, therefore, we investigated whether the stable SS analog [¹²⁵I-Tyr³]octreotide is internalized by SS-R-positive AtT20/D16V mouse pituitary tumor cells and human GH-secreting pituitary tumor cells. We recently found that accumulation of [¹¹¹In-DTPA-D-Phe¹]octreotide in SS-R-positive organs showed a tissue-specific bell-shaped function of the injected mass of the radiopharmaceutical (18). To investigate the cellular mechanisms underlying these observations, we also studied the effect of different concentrations of unlabeled octreotide on binding and internalization of [¹²⁵I-Tyr³]octreotide by the two cell culture systems.

Materials and Methods

Cell culture

AtT20/D16V mouse pituitary tumor cells were obtained from Dr. J. Tooze (European Molecular Biology Organization, Heidelberg, Germany). The cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with sodium pyruvate (1 mM), 10% fetal calf serum, fungizone (0.5 mg/liter), penicillin (10⁵ U/liter), and sodium bicarbonate (2.2 g/liter, final concentration). The medium was adjusted to pH 7.4 with 1 N NaOH. The cells were passaged once a week using trypsin (0.05%) and EDTA (0.02%). For internalization experiments, the cells were seeded at a density of 0.5 × 10⁶ cells/well in 12-well multiwell plates (Costar, Cambridge, MA) and grown to confluency for 2 days.

Human GH-secreting pituitary tumor tissue from seven acromegalic patients was obtained by transphenoidal operation. A single tumor cell suspension was prepared by enzymatic dispersion of the tissue, as described in detail previously (19). For internalization experiments, the tumor cells were cultured for 4 days at a density of 10⁶ cells/3 ml in 12-well multiwell plates (Costar) in Minimal Essential Medium supplemented with nonessential amino acids, sodium pyruvate (1 mM), 10% fetal calf serum, penicillin (10⁵ U/liter), fungizone (0.5 mg/liter), L-glutamine (2 mM), and sodium bicarbonate (2.2 g/liter, final concentration). The medium was adjusted to pH 7.4 with 1 N NaOH.

The effect of 10 nM octreotide on GH release was investigated in all GH-secreting pituitary adenoma cultures (10⁵ cells/well). Experimental conditions and determination of the GH concentration in the medium by immunoradiometric assay were described previously (20).

All cells were cultured at 37 C in a water-jacketed incubator in humidified air with 5% CO₂. The media and supplements were obtained from GIBCO (Paisley, UK).

Radioligands

The SS analog [Tyr³]octreotide (Sandoz, Basel, Switzerland) was iodinated with ¹²⁵I by the chloramine-T method and purified by HPLC, as described previously in detail (21). The specific radioactivity of the radioligand was approximately 2000 Ci/mmol.

Internalization experiments

On the day of the experiment, AtT20 cells were washed twice with internalization medium. The internalization medium consisted of Dulbecco's Modified Eagle's Medium supplemented with HEPES (30 mM), L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (10⁵ U/liter), fungizone (0.5 mg/liter), and 0.2% BSA (fraction V, Sigma Chemical Co., St. Louis, MO). The medium was adjusted to pH 7.4 with 1 N KOH. The cells were allowed to adjust to the medium for 1 h at 37 C. Thereafter, approximately 200,000 cpm [¹²⁵I-Tyr³]octreotide (~0.1 nM, final concentration) were added to the medium, and the cells were incubated at 37 C (or other temperatures when indicated) for a period of up to 4 h in quadruplicate without or with excess unlabeled octreotide (1 μM) to

determine nonspecific membrane binding and internalization. Binding and internalization of [¹²⁵I-Tyr³]octreotide were blocked by excess unlabeled octreotide (Sandoz Pharma, Basel, Switzerland), whereas unrelated peptides, such as LHRH (1 μM; Hoechst, Amsterdam, The Netherlands) and TRH (1 μM; Hoechst), had no effect. In some experiments the cells were pretreated with pertussis toxin (Sigma) for 18 h and with chloroquine (Sigma) or NH₄Cl for 30 min.

Treatment of the cells with low pH was used to distinguish between membrane-bound (acid-releasable) and internalized (acid-resistant) radioligand (22). After the incubation, the cells were washed twice with ice-cold internalization medium. Thereafter, 1 ml sodium acetate (20 mM) in Hanks' Balanced Salt Solution, pH 5.0 (HBSS-Ac), was added to the cells. The cells were then incubated for 10 min at 37 C. After 10 min, the supernatant was collected. Finally, the cells were washed with HBSS-Ac, and the supernatant was pooled with the supernatant of the previous step. This pooled supernatant fraction, acid-extractable radioactivity, represents membrane-bound radioligand. At the end of the incubation, after the HBSS-Ac treatment, the cells were extracted in 1 N NaOH. The radioactivity in this fraction represents internalized radioligand.

In one of the experiments, AtT20 cells were broken by freezing in liquid nitrogen, and the binding characteristics of [¹²⁵I-Tyr³]octreotide to this broken cell preparation were compared to binding and internalization of [¹²⁵I-Tyr³]octreotide by viable AtT20 cells.

Human GH-secreting pituitary tumor cells that had attached to the bottom of the wells during the 4-day culture (see above) were rinsed twice with internalization medium. One milliliter of this medium was then added to the cells, and incubations were performed as described for the AtT20 cells. Both cell types studied remained viable in internalization medium during the 4-h incubation period, as determined by trypan blue exclusion.

Intracellular radioactivity was analyzed by Sep-Pak C₁₈ reverse phase extraction cartridges (Waters Associates, Milford, MA), as described previously (23).

SS-R binding studies

SS-R binding studies were carried out using [¹²⁵I-Tyr³]octreotide as radioligand, as described previously (24). Rat brain cortex or AtT20 cell membrane preparations (corresponding to 15–30 μg protein) were incubated in a total volume of 100 μl at room temperature for 60 min with 30,000–50,000 cpm radioligand and increasing concentrations of unlabeled octreotide in HEPES buffer (10 mM HEPES, 5 mM MgCl₂, and 0.2 g/liter bacitracin, pH 7.6) containing 0.2% BSA (Sigma). After the incubation, 1 ml ice-cold HEPES buffer was added to the assay mixture, and membrane-bound radioactivity was separated from unbound by centrifugation for 2 min at 14,000 rpm in an Eppendorf microcentrifuge. The remaining pellet was washed twice with HEPES buffer, and the final pellet was counted in a γ-counter. Specific binding was taken to be total binding in the absence of excess unlabeled octreotide minus nonspecific binding in the presence of excess (1 μM) unlabeled octreotide. Unrelated compounds (TRH, LHRH, and epidermal growth factor), added in a 1000-fold excess, were not able to displace [¹²⁵I-Tyr³]octreotide binding. Epidermal growth factor was obtained from Saxon Biochemicals (Hannover, Germany).

Analysis of data

Statistical analysis of the data was performed using one-way analysis of variance. When significant overall effects were obtained by analysis of variance, multiple comparisons were made by the Newman-Keuls test (25). The data are expressed as the mean ± SE of at least three independent experiments or as the mean ± SE of four wells per time point or treatment group. IC₅₀ values for displacement of [¹²⁵I-Tyr³]octreotide binding were determined by the computerized program GraphPad [GraphPad, ISI(R) Software, Philadelphia, PA].

Results

Internalization of [¹²⁵I-Tyr³]octreotide by mouse AtT20 pituitary tumor cells

Scatchard analysis of [¹²⁵I-Tyr³]octreotide binding to AtT20 cell membranes revealed a high number of high

affinity SS-R (2815 ± 317 fmol/mg membrane protein; $K_d = 0.35 \pm 0.05$ nM; values are the mean \pm SE of four independent determinations).

To verify that the method of separation of internalized (acid-resistant) and membrane-bound extracellular (acid-releasable) ligand indeed released all binding of radioligand from membrane receptors, rat brain cortex membranes were incubated with [¹²⁵I-Tyr³]octreotide for 1 h at 20 C without or with excess unlabeled octreotide to determine nonspecific binding. Thereafter, the membranes were washed twice with ice-cold binding buffer and subsequently incubated for 10 min at 37 C with HBSS-HAc, as described in *Materials and Methods*. The acid treatment completely abolished specific binding of [¹²⁵I-Tyr³]octreotide, demonstrating that all membrane-bound radioligand is acid releasable.

Figure 1 shows time-dependent increasing specific membrane binding and internalization of [¹²⁵I-Tyr³]octreotide by AtT20 cells. The amount of [¹²⁵I-Tyr³]octreotide internalized was approximately 5% of the dose of radioligand added after 240 min of incubation, and at all time points studied was significantly higher than the amount of radioligand that was membrane bound.

Internalization of [¹²⁵I-Tyr³]octreotide by AtT20 cells was temperature dependent. At 0 C, no binding or internalization of [¹²⁵I-Tyr³]octreotide was seen, whereas the amount of internalized radioligand increased with increasing temperature (20 and 37 C, respectively; Fig. 2).

Displacement of binding of [¹²⁵I-Tyr³]octreotide to AtT20 cells at 37 C showed a bell-shaped curve (Fig. 3, A and B). At lower concentrations of unlabeled octreotide (0.1 and 1 nM), the amounts of both membrane-bound (Fig. 3A) and internalized (Fig. 3B) radioligand were significantly increased compared to those in control cells, whereas at higher concentrations, unlabeled octreotide saturation occurred. In contrast to the observations with viable AtT20 cells, unlabeled octreotide displaced binding of [¹²⁵I-Tyr³]octreotide to a

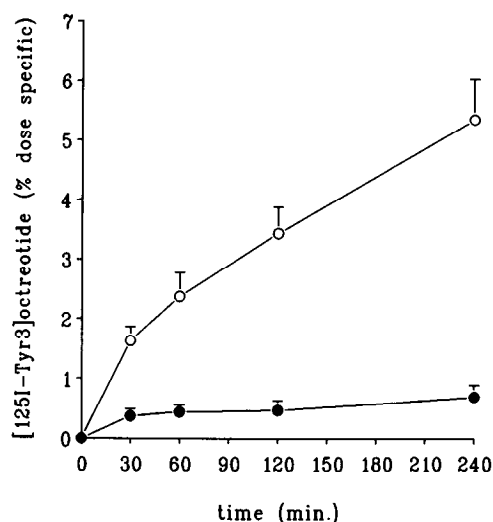


FIG. 1. Time course of membrane binding and internalization of [¹²⁵I-Tyr³]octreotide (●, membrane bound; ○, internalized) by AtT20 mouse pituitary tumor cells. Values are expressed as specific binding and internalization (percentage of the dose of radioligand added) and are the mean \pm SE of three independent experiments.

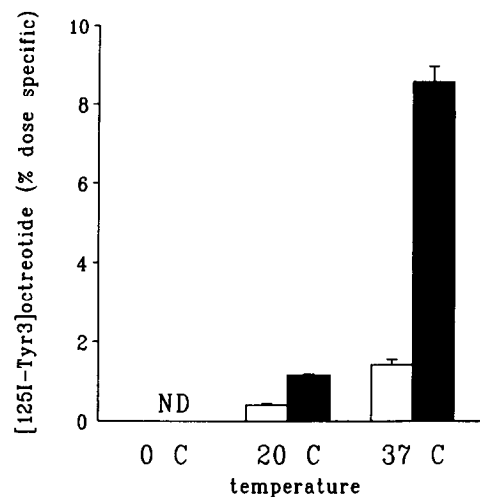


FIG. 2. Temperature dependency of internalization and membrane binding of [¹²⁵I-Tyr³]octreotide by AtT20 mouse pituitary tumor cells. Values are expressed as specific binding and internalization (percentage of the dose of radioligand added) after 4 h of incubation and are the mean \pm SE of four wells per treatment group. □, Membrane-bound radioligand; ■, internalized radioligand. ND, Undetectable.

broken cell preparation of AtT20 cells in a dose-dependent fashion, with an IC_{50} of 0.1 nM (Fig. 3C).

The increased internalization of [¹²⁵I-Tyr³]octreotide induced by this low amount (1 nM) of unlabeled octreotide occurred very rapidly (Fig. 4). Unlabeled octreotide (1 μ M) completely blocked internalization of the radioligand by 15 min of incubation ($P < 0.01$ vs. control cells), whereas 1 nM octreotide nearly doubled internalization at this time point ($P < 0.01$ vs. control cells). Because the increased internalization seems to occur very rapidly, we also studied the effect of 1 nM unlabeled octreotide at several time points between 0–2.5 min of incubation. The results of these experiments are shown in Fig. 5. Again, 1 nM unlabeled octreotide induced a very rapid (within 1 min of incubation) 2-fold increase in the amount of internalized radioligand (Fig. 5, lower panel). At the same time, the amount of membrane-bound [¹²⁵I-Tyr³]octreotide was significantly increased (Fig. 5, upper panel).

To further investigate the mechanism of internalization of [¹²⁵I-Tyr³]octreotide, AtT20 cells were preincubated with pertussis toxin or inhibitors of lysosomal degradation, chloroquine and NH_4Cl . Table 1 shows that pertussis toxin significantly inhibited both membrane binding and the subsequent internalization of [¹²⁵I-Tyr³]octreotide, indicating that this process acts via a pertussis toxin-sensitive GTP-binding protein-dependent mechanism. Chloroquine, an inhibitor of lysosomal degradation, significantly inhibited internalization of the radioligand. NH_4Cl , another inhibitor of lysosomal function, did not significantly affect internalization, however (data not shown). It seems, therefore, that there is no major degradation of the radioligand after 4 h of incubation. This is further substantiated by analysis of intracellular radioactivity by Sep-Pak C_{18} reverse phase cartridges in cells that had been incubated for 4 h with [¹²⁵I-Tyr³]octreotide. This showed that 88% of the radioactivity within the cells was still peptide bound.

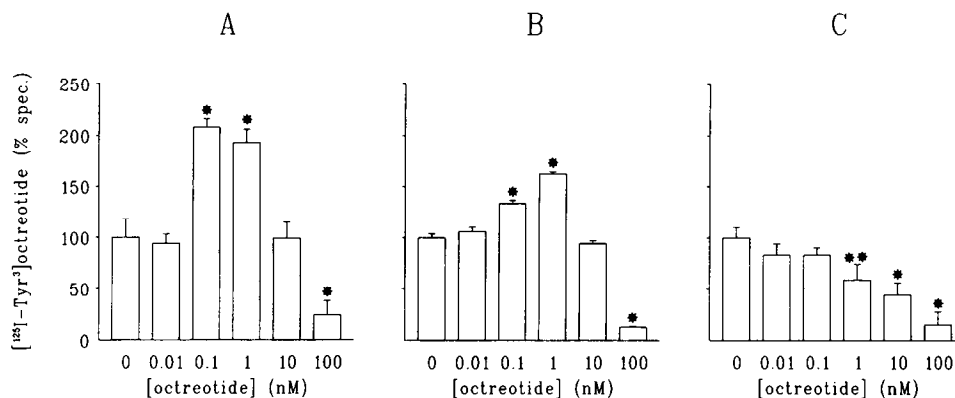


FIG. 3. Bell-shaped function of displacement by increasing concentrations of unlabeled octreotide of membrane-bound (A) and internalized (B) [¹²⁵I-Tyr³]octreotide by AtT20 pituitary tumor cells and displacement of binding of [¹²⁵I-Tyr³]octreotide by unlabeled octreotide to a broken cell preparation of AtT20 cells (C). In the experiment shown in C, the cells were broken by freezing in liquid nitrogen before incubation with [¹²⁵I-Tyr³]octreotide under the same conditions as in A and B. Values are expressed as a percentage of specific binding or internalization in control cells and are the mean \pm SE of four wells per treatment group. Values for control cells were $0.16 \pm 0.4\%$ (A), $4.02 \pm 0.15\%$ (B), and $1.91 \pm 0.49\%$ (C) of the dose of radioligand added. **, $P < 0.05$; *, $P < 0.01$ [vs. control cells (0 nM)].

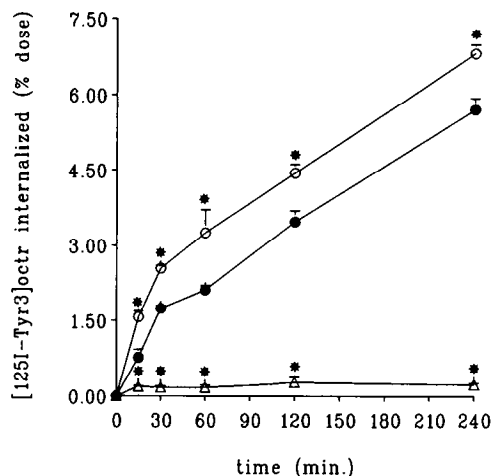


FIG. 4. Effect of $1 \mu\text{M}$ and 1 nM unlabeled octreotide on internalization of [¹²⁵I-Tyr³]octreotide by mouse AtT20 pituitary tumor cells. *, $P < 0.01$, 1 nM or $1 \mu\text{M}$ octreotide vs. control cells. Values are expressed as a percentage of the dose of radioligand added and are the mean \pm SE of three independent experiments. ●, Control cells; Δ , $1 \mu\text{M}$ octreotide; ○, 1 nM octreotide.

Internalization of [¹²⁵I-Tyr³]octreotide by human GH-secreting pituitary adenoma cells

Similar to the observations in AtT20 cells, human GH-secreting pituitary adenoma cells from patient 1 showed a time-dependent specific membrane binding (Fig. 6, upper panel) and internalization of [¹²⁵I-Tyr³]octreotide (Fig. 6, lower panel). In the presence of 1 nM unlabeled octreotide, a rapid increase in both membrane-bound and internalized radioligand was observed. A statistically significant higher amount of radioligand was bound and internalized at all time points studied. After 60 min of incubation, an approximately 6-fold higher amount of radioligand was bound and internalized in the presence of 1 nM unlabeled octreotide compared to that in control cells (specific binding of control cells, 0.2% vs. 1.3% at 1 nM octreotide; specific internalization by control cells, 0.4% vs. 2.5% at 1 nM octreotide). The total amounts of radioligand bound and

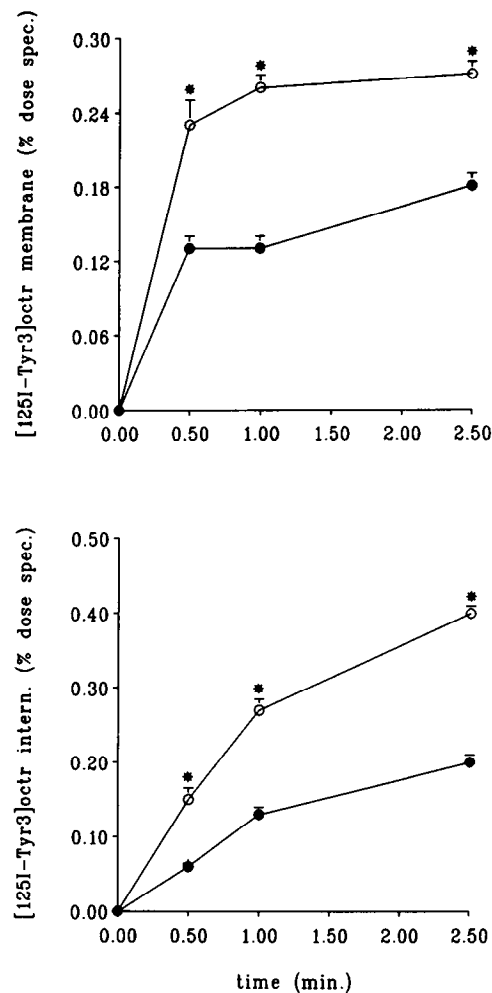


FIG. 5. Rapid increase in membrane binding (upper panel) and internalization (lower panel) of [¹²⁵I-Tyr³]octreotide induced by 1 nM unlabeled octreotide. ●, Control; ○, 1 nM unlabeled octreotide. *, $P < 0.01$ vs. control cells. Values are expressed as specific binding and internalization (percentage of the dose of radioligand added) and are the mean \pm SE of three independent experiments.

TABLE 1. The effects of pertussis toxin (PT) and chloroquine on binding and internalization of [¹²⁵I-Tyr³]octreotide by mouse AtT20 pituitary tumor cells

	[¹²⁵ I-Tyr ³]Octreotide (% of dose)	
	Membrane bound	Internalized
Control	0.30 ± 0.04	6.39 ± 0.15
PT (100 µg/liter)	0.14 ± 0.03 ^a	3.47 ± 0.05 ^a
PT (500 µg/liter)	0.14 ± 0.04 ^a	2.93 ± 0.19 ^a
Chloroquine (100 µM)	0.37 ± 0.02	2.12 ± 0.10 ^a

The cells were pretreated for 16 h with PT and for 30 min with chloroquine. Thereafter, the cells were incubated for 4 h with 0.1 nM [¹²⁵I-Tyr³]octreotide without or with the test substances. Parallel incubations with excess unlabeled octreotide (1 µM) were performed to determine nonspecific binding and internalization. Separation of membrane-bound and internalized radioligand was performed as described in *Materials and Methods*. Values represent specific binding and internalization (percentage of the dose of radioligand added) and are the mean ± SE of four wells per treatment group.

^a *P* < 0.01 vs. control.

internalized by these GH-secreting pituitary adenoma cells after 4-h incubation were also high (1.28% and 4.98% of the dose of radioligand added, respectively).

The effect of 1 nM unlabeled octreotide was further investigated in six other GH-secreting adenoma cell cultures. Table 2 shows that the amount of internalized radioligand was significantly increased (32–122% increase) by 1 nM octreotide in five of six cultures (no. 1, 4, 5, 6, and 7), which showed internalization of the radioligand, whereas the amount of membrane-bound radioligand was simultaneously increased in three of six cultures (no. 1, 6, and 7; 30–113% increase). Cultured cells from patient 3 showed no binding and internalization, whereas 1 nM octreotide significantly lowered internalization by the cultured cells of patient 2. Comparable to the findings in AtT20 cells, displacement of internalization of [¹²⁵I-Tyr³]octreotide by cells of patient 4 showed a bell-shaped curve, with a maximal increase by 1 nM unlabeled octreotide and saturation at higher octreotide concentrations (not shown).

Interestingly, we found that the preoperative sc administration of 50 µg octreotide induced a significant lowering of serum GH levels in patients 1 and 2, whereas patients 3 and 4 showed no response of GH levels to octreotide *in vivo* (Fig. 7). In support of this sensitivity to octreotide *in vivo*, we observed that GH release by cultured cells from patients 1 and 2 was significantly more sensitive to octreotide *in vitro* than that by cultured tumor cells from patients 3 and 4. GH release by cultured tumor cells from patients 1, 2, 3, and 4 was inhibited by octreotide (10 nM) by 83 ± 9%, 43 ± 2%, 27 ± 1%, and 16 ± 0%, respectively. GH release by cells of patients 5, 6, and 7 was inhibited by 43 ± 1%, 25 ± 1%, and 27 ± 1%, respectively. Thus, the cells of patients 1 and 5, which showed a high amount of internalization of [¹²⁵I-Tyr³]octreotide, also showed a high responsiveness of GH release to octreotide *in vitro*.

Discussion

A variety of human neuroendocrine tumors contain receptors for the tetradecapeptide SS (1). The presence of SS-R on these tumors enabled us to develop a technique that allows *in*

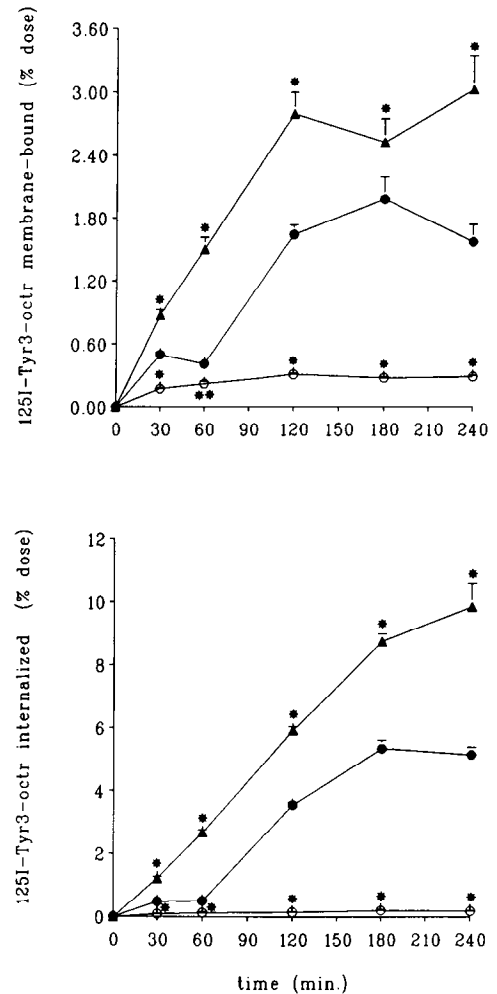


FIG. 6. Effects of 1 µM and 1 nM unlabeled octreotide on membrane binding (upper panel) and internalization (lower panel) of [¹²⁵I-Tyr³]octreotide by human GH-secreting pituitary adenoma cells of patient 1. **, *P* < 0.05; *, *P* < 0.01 (vs. control cells). Values are expressed as a percentage of the dose of radioligand added and are the mean ± SE of four wells per treatment group. ●, Control cells; ○, 1 µM octreotide; ▲, 1 nM octreotide

in vivo visualization in man of SS-R-positive tumors using the radiolabeled SS analogs [¹²⁵I-Tyr³]octreotide and [¹¹¹In-DTPA-D-Phe¹]octreotide (2–6). In several tumors (*i.e.* carcinoids and paragangliomas) that express a high amount of SS-R, binding of radioactivity *in vivo* exceeds estimates of 0.1% of the administered dose per g tumor tissue. Radiotherapy using SS analogs coupled to α- or β-emitting isotopes has been proposed as an application for radiolabeled SS analogs and has recently been carried out in one patient (5, 6, 26). To develop this concept further, it is of importance to know to what extent the above-mentioned radiolabeled SS analogs are internalized by SS-R-positive tumor cells, and whether it is possible to manipulate the degree of internalization.

In the present study we demonstrated a specific time- and temperature-dependent high amount of internalization of [¹²⁵I-Tyr³]octreotide by mouse AtT20 pituitary tumor cells. Binding and internalization were inhibited by pertussis toxin, demonstrating that a pertussis toxin-sensitive GTP-binding protein is involved in these processes. A previous

TABLE 2. The effect of 1 nM unlabeled octreotide (Octr) on the amount of membrane-bound and internalized [¹²⁵I-Tyr³]octreotide by human GH-secreting pituitary adenoma cell cultures

Patient No.	Treatment	[¹²⁵ I-Tyr ³]Octr (% of dose)	
		Membrane bound	Internalized
1	Control	1.28 ± 0.18	4.98 ± 0.35
	Octr (1 nM)	2.73 ± 0.33 ^a	9.66 ± 0.73 ^a
2	Control	0.48 ± 0.02	0.60 ± 0.00
	Octr (1 nM)	0.43 ± 0.11	0.40 ± 0.04 ^a
3	Control	Not detectable	Not detectable
	Octr (1 nM)	Not detectable	Not detectable
4	Control	0.08 ± 0.00	0.41 ± 0.02
	Octr (1 nM)	0.09 ± 0.00	0.58 ± 0.02 ^a
5	Control	0.58 ± 0.02	2.46 ± 0.14
	Octr (1 nM)	0.67 ± 0.08	3.25 ± 0.17 ^a
6	Control	0.13 ± 0.02	0.24 ± 0.03
	Octr (1 nM)	0.22 ± 0.01 ^a	0.51 ± 0.05 ^a
7	Control	0.23 ± 0.01	0.73 ± 0.02
	Octr (1 nM)	0.30 ± 0.01 ^a	1.62 ± 0.03 ^a

GH-secreting pituitary adenoma cells (10⁶) from seven acromegalic patients were incubated for 4 h with 0.1 nM [¹²⁵I-Tyr³]Octr without or with 1 nM unlabeled Octr. Parallel incubations with excess unlabeled Octr (1 μM) were performed to determine nonspecific binding and internalization. Separation of membrane-bound and internalized radioligand was performed as described in *Materials and Methods*. Values represent specific binding and internalization (percentage of the dose of radioligand added) and are the mean ± SE of four wells per treatment group.

^a *P* < 0.01 vs. control.

study showed that pertussis toxin reduced the affinity of SS-R for SS (and its structural analogs) in AtT20 cells (27). In addition, internalization of [¹²⁵I-Tyr³]octreotide by human GH-secreting pituitary tumor cells was found. Several studies have demonstrated receptor-mediated endocytosis of SS in rat anterior pituitary cells (7, 9, 11, 12), rat islet cells (10, 14), and AtT20 cells (8). Other investigators, however, showed that [¹²⁵I-Tyr¹]SS-14 and [¹²⁵I-Tyr¹¹]SS-14, unlike [¹²⁵I]epidermal growth factor, are not rapidly internalized by GH₄C₁ rat pituitary cells and RINm5F insulinoma cells, respectively (15, 16). These investigators suggested that the radioligands they used were rapidly degraded by membrane proteases. From our present study, it is clear that a high amount of the radioiodinated SS analog Tyr³-octreotide is internalized by AtT20 mouse and human GH-secreting pituitary tumor cells. This is probably related to the high stability of [¹²⁵I-Tyr³]octreotide (17). The parental cyclic octapeptide analog octreotide (SMS 201-995) was shown to be highly resistant to degradation by pure enzymes and tissue homogenates (28). In agreement with this, we found that most of the radioactivity present in the cells after 4 h of incubation was still peptide bound, although we did not perform HPLC analysis to demonstrate that the peptide-bound radioactivity represented intact radioligand. In addition, inhibitors of lysosomal degradation (chloroquine and NH₄Cl) did not increase the amount of acid-resistant (internalized) radioactivity. In fact, in agreement with a previous study by Presky and Schonbrunn (15), chloroquine

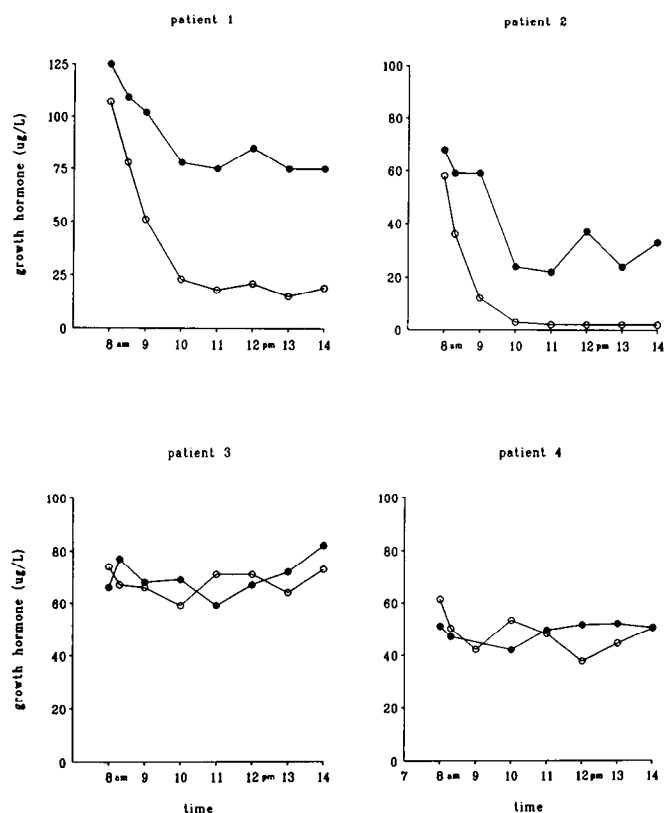


FIG. 7. Effect of the administration of 50 μg octreotide, sc, on serum GH levels in four acromegalic patients. ●, Placebo at 0815 h; ○, 50 μg octreotide, sc, at 0815 h.

significantly inhibited the amount of internalized radioligand. It appears, therefore, that the inhibitory effect of chloroquine is due to direct interference with the process of receptor-mediated endocytosis, and that this latter effect prevails over its inhibitory effect on lysosomal function under the conditions of our study. Our data favor further development of the concept of radiotherapy using radiolabeled SS analogs.

Another finding of our present study is the rapidly increasing binding and internalization of [¹²⁵I-Tyr³]octreotide when AtT20 cells and human GH-secreting adenoma cells are simultaneously incubated with a low concentration of unlabeled octreotide. Displacement of binding and internalization of radioligand by AtT20 cells by unlabeled octreotide showed a bell-shaped curve at 37 C. An increase was found at low unlabeled octreotide concentrations (0.1 and 1 nM), whereas at higher concentrations, saturation was observed. In contrast to this, displacement of binding by unlabeled octreotide to a broken cell preparation of AtT20 cells showed, under the same incubation conditions, a dose-dependent inhibition curve with an IC₅₀ of 0.1 nM, in agreement with the estimated K_d of the SS-R on these cells. This suggests that binding of [¹²⁵I-Tyr³]octreotide to viable cells is a dynamic process and may, therefore, show binding characteristics different from those of binding to broken cells or a membrane homogenate preparation.

The increased internalization occurred very rapidly. Within 1 min of incubation, 1 nM unlabeled octreotide caused

an increase in the amount of binding and internalization of [¹²⁵I-Tyr³]octreotide by AtT20 cells. In addition, increased internalization in the presence of 1 nM unlabeled octreotide was found in five of six cultures of human GH-secreting pituitary tumors. In three of these human GH-secreting adenoma cell cultures, this increased internalization was accompanied by an increase in the amount of membrane-bound radioligand, which suggests a very rapid increase in the number of SS-R on these cells.

There are several studies concerned with the homologous regulation of SS-R expression. In GH₄C₁ cells, Presky and Schonbrunn (22) demonstrated a time-dependent increase in [¹²⁵I-Tyr¹]SS-14 binding by the addition of unlabeled SS-14, which reached a maximum of approximately 200% after 20 h. Other investigators (29, 30) showed a decrease in SS binding by AtT20 cells after preincubation for 4 h with SS-14 or SS-28. In 7315b rat prolactinoma cells, chronic exposure (up to 5 weeks) results in a reversible down-regulation of SS-R (31). In an earlier study, Schonbrunn and Tashjian (32) showed in GH₄C₁ cells a dose-dependent displacement of binding of [¹²⁵I-Tyr¹]SS by unlabeled SS. Therefore, it has to be established whether apart from AtT20 and human GH-secreting pituitary adenoma cells, other cell types respond to unlabeled octreotide with an increased binding and internalization of radioiodinated [Tyr³]octreotide. In our study we also found that unlabeled SS-14 and SS-28 induced this increase in AtT20 cells (data not shown).

At present, we have no explanation for the mechanism of the increased binding and subsequent internalization of [¹²⁵I-Tyr³]octreotide by AtT20 tumor cells and human GH-secreting adenoma cells induced by simultaneous incubation with a low concentration of unlabeled octreotide. The rapid increase in [¹²⁵I-Tyr³]octreotide binding and internalization seems unlikely to be caused by *de novo* synthesis of SS-R, because this increase occurred within several minutes of incubation with 1 nM unlabeled octreotide. Therefore, the most likely explanation for this phenomenon is a rapid recruitment of cellular SS-R to the outside of the cell membrane, although an increased internalization rate or an effect of octreotide on SS-R synthesis cannot be fully excluded. In this respect, it should be mentioned that glucose has been shown to increase the amount of internalized SS in pancreatic islets, probably due in part to the increase in the cell surface SS-R concentration by migration of the secretion vesicles during endocytosis, promoting increased transport of SS-R (33). Short term pretreatment of AtT20 cells with 1 nM octreotide did not result in a significant change in the estimated K_d of [¹²⁵I-Tyr³]octreotide binding (our unpublished observation), indicating that the increased internalization is not caused by an augmented affinity of the receptor.

Recently, at least five different SS-R subtypes have been cloned. All subtypes bind SS-14 and SS-28 with high affinity, whereas their affinities for numerous SS analogs differ considerably (34–39). Octreotide binds with high affinity to the human SSTR2 (hSSTR2) subtype, whereas this analog has a moderate affinity to hSSTR3 and hSSTR5 subtypes and shows no binding to the hSSTR subtypes 1 and 4 (34–39). More than one hSSTR subtype is expressed in human GH-secreting adenomas. Greenman and Melmed (40, 41) showed in 3 of 7, 9 of 10, 6 of 7, 0 of 11, and 10 of 11 human

GH-secreting adenomas, expression of hSSTR1, hSSTR2, hSSTR3, hSSTR4, and hSSTR5, respectively. In addition, Reubi *et al.* (42) recently demonstrated in GH-secreting adenomas, by *in situ* hybridization autoradiography, hSSTR2 (7 of 7 cases) and hSSTR3 (3 of 7), but not hSSTR1, expression. In all cases, binding of [¹²⁵I-Tyr³]octreotide and [¹²⁵I-Leu⁸, D-Trp²², Tyr²⁵]SS-28 was found (42). In AtT20 cells, four SSTR genes (subtypes 1, 2, 4, and 5) are expressed, of which SSTR2 expression predominates (43). At present, it is unclear which SSTR subtype is involved in receptor-mediated endocytosis by human GH-secreting adenoma and AtT20 cells. Bruno and Berelowitz (44) recently demonstrated that chronic SS exposure of GH₃ cells increased specific [¹²⁵I-Tyr¹]SS binding, whereas SS also increased SSTR1–5 messenger RNA (mRNA) expression in these cells. Although SSTR1, -3, -4, and -5 mRNA increased after 6–48 h of exposure to SS, SSTR2 mRNA showed a biphasic response, initially increasing at 2 h, then decreasing at 6 h, and achieving normalization by 48 h. These data suggest that the increased binding and internalization of [¹²⁵I-Tyr³]octreotide after 4 h of exposure to unlabeled octreotide may also be caused by an increase in SSTR2 subtype expression in AtT20- and human GH-secreting adenoma cells. Studies on internalization of [¹²⁵I-Tyr³]octreotide by cell lines stably expressing SSTR subtypes may help to answer this question. If the SSTR1 and SSTR4 subtypes, to which octreotide does not bind, also show ligand-induced internalization, SS analogs with high affinity to these SSTR subtypes may be of interest for radiotherapeutic application as well. However, such analogs are not available for clinical use at present.

Our observation of a higher amount of binding and internalization of [¹²⁵I-Tyr³]octreotide when the cells are simultaneously incubated with a low concentration of unlabeled octreotide may explain the results of a recent *in vivo* study by our group, in which we found that uptake of [¹¹¹In-DTPA-D-Phe¹]octreotide in SS-R positive organs (pituitary, pancreas, and adrenals) in rats showed a bell-shaped function of the injected mass (18). Therefore, the highest specific radioactivity may not automatically result in the highest binding and subsequent internalization of the radioligand by the tumor in *in vivo* SS-R imaging using radiolabeled SS analogs in patients with SS-R-positive tumors. Indeed, preliminary findings in patients indicate that scintigraphy with a specific activity higher than 220 megabecquerels ¹¹¹In/5 μg [DTPA-D-Phe¹]octreotide will lead to decreased quality, and uptake in tumors will be significantly reduced (45). Moreover, Dörr *et al.* (46) recently demonstrated an improved visualization of carcinoid liver metastases in patients by [¹¹¹In-DTPA-D-Phe¹]octreotide during octreotide treatment, which might be due to up-regulation of SS-R by octreotide. Finally, it should be mentioned that we have used [¹²⁵I-Tyr³]octreotide as a radioligand in our study. It has yet to be established, however, whether radiolabeled DTPA-conjugated SS analogs, such as [¹¹¹In-DTPA-D-Phe¹]octreotide, are internalized by SS-R-positive tumor cells to the same extent as non-DTPA-coupled SS analogs.

In conclusion, a high amount of [¹²⁵I-Tyr³]octreotide is internalized in a specific time-, temperature-, and pertussis toxin-sensitive GTP-binding protein-dependent manner by mouse AtT20 cells and human GH-secreting pituitary tumor

cells. The amount of internalized [¹²⁵I-Tyr³]octreotide by the human GH-secreting adenoma cell cultures correlated well with their *in vivo* and *in vitro* responsiveness of GH secretion to octreotide. Simultaneous incubation with a low concentration of unlabeled octreotide induces a very rapid increase in membrane binding and in the amount of internalization of [¹²⁵I-Tyr³]octreotide by AtT20 cells. Five of six human GH-secreting adenoma cell cultures also responded to unlabeled octreotide with an increase in the amount of internalized radioligand. It remains to be established, however, whether our present conclusions also apply to other types of human SS-R-positive neuroendocrine tumors.

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