EXPERIMENTAL STUDY

Somatostatin and its analog lanreotide inhibit the proliferation of dispersed human non-functioning pituitary adenoma cells *in vitro*

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Abstract

Objective: Somatostatin is a powerful inhibitor of hormone secretion and cell proliferation. Treatment with somatostatin analogs in humans causes a reduction in size and secretory activity of some endocrine tumors, including somatotrophic pituitary adenomas. Less studied are the effects of somatostatin agonists on non-functioning pituitary adenomas (NFPAs). In this study we characterized the effects of somatostatin and its analog lanreotide on the proliferation of NFPAs *in vitro* and the intracellular mechanisms involved.

Design: Twenty-three NFPA post-surgical specimens were analyzed for somatostatin receptor (SSTR) expression and 12 of them were cultured *in vitro* to study somatostatin's effects on cell proliferation, assessed by means of [³H]thymidine uptake, and the intracellular signaling.

Results: One or more SSTR subtypes were expressed in 90% of the adenomas tested. Somatostatin and lanreotide treatment inhibited phorbol myristate acetate (PMA)-induced cell proliferation. Vanadate pretreatment reversed somatostatin and lanreotide inhibition of PMA-induced DNA synthesis suggesting an involvement of tyrosine phosphatase in this effect. In the only adenoma tested, somatostatin directly induced a tyrosine phosphatase activity. Somatostatin and lanreotide caused also a significant inhibition of voltage-sensitive calcium channel activity induced by 40 mmol/l K⁺ depolarization in microfluorimetric analysis.

Conclusions: These data show that somatostatin and lanreotide inhibit human NFPA cell proliferation *in vitro*, and suggest that activation of tyrosine phosphatases and inhibition of the activity of voltage-dependent calcium channels may represent intracellular signals mediating this effect.

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Introduction

Pituitary tumors are classified mainly by the characteristic clinical syndromes that accompany tumor hormone production. About 25–30% of pituitary adenomas are non-functioning (1). The definition of non-functioning pituitary adenoma (NFPA) refers to strictly clinical features of these tumors, since immunohistochemistry studies demonstrate that the majority of NFPAs do synthesize and sometimes secrete hormones. In most cases the produced substances are subunits of glycoprotein hormones (luteinizing hormone (LH), follicle-stimulating hormone (FSH) and thyrotropin), mainly the common α -subunit (1), but occasionally also intact glycoprotein hormones have been reported to

be produced (2, 3). About 17% of NFPAs are pure 'null' adenomas, likely derived from neoplastic transformation of hormonally inactive transitional, undifferentiated or precursor cells, identified in normal pituitaries (1, 4).

Somatostatin is a peptide hormone that displays a range of biological actions, including inhibition of hormone secretion, modulation of neuronal transmission and regulation of cell growth (4). Somatostatin acts through a family of G protein-coupled receptors variably expressed in both normal tissues and tumors (5). Upon activation of these receptors, somatostatin inhibits cell proliferation in many epithelial and endocrine cells (6–9), through a direct antagonism on the activity of epidermal growth factor (EGF) in pancreatic tumor cells (10), and related tyrosine kinases in other cell

types (7, 11). The inhibition of tyrosine kinase activity was reported to be a consequence of the ability of somatostatin to stimulate a phosphotyrosine phosphatase (PTP) activity (7, 12–15). One proposed target of this phosphatase is the EGF receptor itself as demonstrated both *in vitro* and *in vivo* (14, 15).

The presence of all the five cloned somatostatin receptor (SSTR) subtypes in the human fetal normal pituitary (16), as well as in the rat pituitary (17), has been reported. Moreover, it was demonstrated that each single cell type in the rat pituitary expresses more than one SSTR subtype (18). In NFPA the expression of the SSTRs is conserved, with a prevalence of the subtypes SSTR2 and SSTR3, although a certain variability among the different studies was reported (19–21).

Although previous clinical studies showed in only a limited number of cases a positive effect of pretreatment of NFPA with somatostatin analogs, in terms of tumor shrinkage, reduction of α -subunit secretion and visual improvement (22–24), the better understanding of the intimate mechanisms involved in the antiproliferative effects of somatostatin and the molecular cloning of a whole family of SSTRs led us to investigate the capability of somatostatin and its analog lanreotide to inhibit the proliferation of human NFPAs *in vitro* and try to correlate these effects with the expression of the different SSTR subtypes. Moreover, we analyzed the possible intracellular mechanisms involved, evaluating both the participation of PTPs and the inhibitory effects on

the depolarization-dependent voltage-sensitive calcium channel activity.

Materials and methods

Patients

Twenty-three human NFPA surgical specimens were obtained from the Neurosurgery Department, School of Medicine, University of Genoa. Tumor characteristics are described in Table 1. Fifteen patients were male (65%) and eight female (35%); the age ranged from 32 to 76 with an average of 54.9 years. Twelve surgical specimens (P1–P12) were divided into two pieces: one was immediately frozen for SSTR mRNA expression and the other dispersed to single cells for culturing *in vitro* (see below) and analyzed for cell proliferation and second messenger regulation (P1 and P3 were too small, therefore they were only analyzed for *in vitro* studies). Eleven (P13–P23) of these adenomas were immediately frozen at -80°C and subsequently analyzed for SSTR mRNA expression. To avoid contamination with normal pituitary cells only the central portion of the adenomas was used for the experiments.

Primary cultures of pituitary adenoma cells

Post-surgical specimens were washed in buffer (15 mmol/l Hepes, pH 7.4, 137 mmol/l NaCl, 5 mmol/l

Table 1 Non-functioning adenomas: patient data.

	Sex	Age	Tumour extension	Pre-operative therapy	LH		FSH		α -subunit	
					(pre)	(post)	(pre)	(post)	(pre)	(post)
P 1	M	66	Extrasellar (macroadenoma)	–	<1	<1	1	<1	0.4	nd
P 2	M	41	Extrasellar (macroadenoma)	–	na	na	na	na	na	na
P 3	M	76	Intrasellar	–	<1	<1	<1	6	0.2	0.1
P 4	M	45	Intrasellar	DEX	3	3	<1	1	0.7	0.4
P 5	F	61	Extrasellar (recurrent macroadenoma)	–	<1	<1	0.5	0.7	1	0.5
P 6	F	59	Intrasellar	–	<1	<1	<1	<1	0.5	0.6
P 7	M	51	Extrasellar	–	1	1	2	6	0.4	nd
P 8	M	63	Intrasellar	–	5	4	6	4	0.4	nd
P 9	F	44	Intrasellar	–	6	7	7	9	nd	0.3
P10	F	63	Extrasellar (recurrent)	–	<1	<1	<1	<1	1	1
P11	M	41	Extrasellar (invasive macroadenoma)	–	<1	2	1	1	1	1
P12	M	59	Extrasellar (recurrent macroadenoma)	RX/BC	<1	<1	4	<1	nd	nd
P13	M	52	Extrasellar	–	<1	<1	<1	3	0.5	nd
P14	M	61	Extrasellar	–	na	na	na	na	na	na
P15	M	47	Extrasellar (invasive)	–	0.5	0.04	0.3	1.9	nd	nd
P16	F	32	Intrasellar (recurrent)	–	6.6	nd	12.3	nd	nd	nd
P17	M	60	Extrasellar	–	3	2	5	8	3.6	0.2
P18	M	69	Intrasellar	–	<1	nd	3	nd	0.2	nd
P19	F	32	Intrasellar	–	na	na	na	na	na	na
P20	F	44	Extrasellar (recurrent)	–	na	na	na	na	na	na
P21	M	72	Intrasellar	–	2	<1	4	2	0.2	nd
P22	F	64	Intrasellar	–	na	na	na	na	na	na
P23	M	61	Extrasellar	–	<1	<1	na	38	6.6	2.3

LH, FSH and α -subunit in mIU/ml (pre = pre-operative; post = post-operative).

na = not available; nd = not done, BC = bromocriptine; DEX = dexamethasone; RX = radiotherapy.

KCl, 0.7 mmol/l Na_2HPO_4 , 10 mmol/l glucose, 2.5 mg/ml amphotericin B and 10^5 U/l penicillin/streptomycin) and immediately divided into two pieces. The first was processed for RNA extraction and the other was mechanically dispersed in the same buffer for *in vitro* experiments. Average yield was about $3\text{--}5 \times 10^6$ viable cells/adenoma. Isolated cells were plated at a density of 100 000/well in poly-L-lysine-coated 24-well plates for proliferation analysis, or on poly-L-lysine-coated coverslips for microfluorimetry studies.

Cells were grown in minimal essential medium containing D-valine instead of L-valine, to prevent fibroblast proliferation (25, 26), 10% fetal calf serum (FCS) and insulin (5 mg/l), transferrin (5 mg/l), sodium selenite (20 mg/l) and non-essential amino acids.

mRNA analysis

The expression of specific mRNAs was evaluated by means of the reverse transcriptase (RT)-PCR technique. Total RNA was isolated using the acidic phenol technique (27). RNA integrity was analyzed by formaldehyde agarose gel electrophoresis. Total RNA was treated for 45 min with RNase-free DNase and then phenol/chloroform extracted and ethanol precipitated. cDNA was synthesized using AMV RT (Finnzyme OY, Finland) and oligo-dT(16) primers. Ten nanograms cDNA were subsequently used in the PCR reaction for 35 cycles (1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C, followed by 7 min at 72 °C). Amplified DNA fragments were then visualized on 1.5% agarose gel electrophoresis. The primers used for SSTR1, SSTR3, SSTR4 and SSTR5 were previously described (19). For SSTR2 the 5'-sense primer corresponded to the amino acids 71–77 and 3'-antisense primer corresponded to the amino acids 195–181 of the human SSTR2 sequence; expected lengths for the amplified products were the following: SSTR1 = 401 bp, SSTR2 = 392 bp, SSTR3 = 222 bp, SSTR4 = 321 bp, SSTR5 = 223 bp. The efficiency of the PCR reaction was evaluated by amplifying a β -actin fragment using specific oligo-primers.

Proliferation assay

DNA synthesis activity was measured by means of the [^3H]thymidine uptake assay as previously reported (28). Briefly, cells were plated at a density of 1×10^5 in 24-well plates. After 24 h cells were serum- and growth factor-starved for 24 h. Subsequently, they were treated with the test substances and 2 $\mu\text{Ci}/\text{ml}$ [^3H]thymidine (Amersham Italia Srl, Milan, Italy) for 16 h. At the end of the incubation time cells were trypsinized (15 min at 37 °C), extracted in 10% trichloroacetic acid (TCA) and filtered under vacuum through fiber glass filters (GF/A; Whatman International, Maidstone, Kent, UK). The filters were then washed sequentially, under vacuum, with 10% and 5% TCA and 95% ethanol.

The radioactivity of the TCA-insoluble fraction was then counted in a scintillation counter.

PTP assay

Cells, plated at 50% confluence in 6 cm Petri dishes, were preincubated with the test substances for 2 h in FCS-free medium at 37 °C in a CO_2 incubator. Then the cells were washed with PBS and mechanically scraped in a buffer containing 0.32 mol/l sucrose, 10 mmol/l Tris, pH 7.5, 5 mmol/l EGTA, 1 mmol/l EDTA and sonicated. Cell lysate was then centrifuged at 15 000 *g* at 4 °C for 60 min, resuspended in a buffer containing 250 mmol/l Hepes, pH 7.2, 140 mmol/l NaCl, 1% NP40 and phenylmethylsulfonyl fluoride and leupeptin as protease inhibitors, and assayed for protein content using the method of Bradford (29) with BSA as standard and the BioRad reagent (BioRad, Segrate, Italy). Twenty micrograms of control or treated membranes were used in the PTP assay, performed using the synthetic substrate para-nitro-phenylphosphate (p-Npp) in a spectrophotometric assay. p-Npp is a general phosphatase substrate that in the presence of inhibitors of serine/threonine phosphatases is specific for PTPs (15, 28). Membranes were preincubated for 5 min at 30 °C in an 80 μl volume containing 20 μl of a $5 \times$ reaction buffer (250 mmol/l Hepes, pH 7.2, 50 mmol/l dithiothreitol, 25 mmol/l EDTA and 500 nmol/l microcystin-leucine-arginine (Alamone Labs., Jerusalem, Israel), as a serine/threonine phosphatases inhibitor). The reaction was started by adding 20 μl 50 mmol/l p-Npp, carried out for 30 min at 30 °C and stopped by adding 900 μl 0.2 mol/l NaOH. The absorbance of the sample, directly proportional to the amount of dephosphorylated substrate, was measured at 410 nm (30). The extinction coefficient for p-Npp at this wavelength is 1.78×10^4 per mol/l per cm (30).

Measurement of intracellular calcium concentrations at single cell level

Cells were plated on 25 mm clean glass coverslips, previously coated with poly-L-lysine (10 $\mu\text{g}/\text{ml}$) and transferred to 35 mm Petri dishes. After 24–48 h cells were serum-starved for a further 24 h. On the day of the experiment the cells were washed for 10 min with a balanced salt solution (Hepes 10 mmol/l, pH 7.4, NaCl 150 mmol/l, KCl 5.5 mmol/l, CaCl_2 1.5 mmol/l, MgSO_4 1.2 mmol/l and glucose 10 mmol/l). Then adenomatous cells were loaded with fura-2 penta-acetoxymethyl ester (fura-2) (4 $\mu\text{mol}/\text{l}$) (Calbiochem-Novabiochem, Lauffentigen, Switzerland) for 60 min at room temperature. Fluorescence measurements were performed as previously reported (31). Briefly, coverslips were mounted on a coverslip chamber and fura-2 fluorescence was imaged with an inverted Nikon diaphot microscope using a Nikon (Firenze, Italy) 40X/1.3 NA Fluor DL objective lens. Cells were illuminated with a

xenon lamp with quartz collector lenses. A shutter and a filter wheel containing the two different excitation filters (340 and 380 nm) were controlled by computer. Emitted light was passed through a 400 nm dichroic mirror, filtered at 490 nm and collected by a digital camera connected with a light intensifier (Applied Imaging, London, UK). Images were digitized and averaged in an image processor connected to a computer equipped with Tardis software (Applied Imaging). For the calibration of fluorescence signals, we used cells loaded with fura-2; $R_{\max}$ and $R_{\min}$ are ratios at saturating and zero intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) respectively, and were obtained perfusing the cells with a salt solution containing CaCl_2 (10 mmol/l), digitonin (2.5 $\mu\text{mol/l}$) and ionomycin (2 $\mu\text{mol/l}$) and subsequently with a Ca^{2+} -free salt solution containing EGTA (10 mmol/l). The values of obtained $R_{\max}$ and $R_{\min}$, expressed as gray level mean, were used to calculate the $[\text{Ca}^{2+}]_i$, using Tardis software, according to the equation of Grynkiewicz *et al.* (32).

Statistics

Experiments were performed in triplicate or quadruplicate and statistical significance analyzed by ANOVA. A *P* value less than 0.05 was considered statistically significant.

Substances

Somatostatin was purchased from Nova-Biochem (Laufentigen, Switzerland) and sodium orthovanadate from ICN Biomedicals Inc. (Aurora, OH, USA), lanreotide was a gift from Beaufur-IPSEN Group (Milford, MA, USA) and all other compounds were from Sigma-Aldrich Srl (Milan, Italy), unless otherwise specified.

Results

SSTR expression

The expression of the five cloned SSTRs was evaluated in 21 NFPA by RT-PCR. Nineteen out of 21 (90%) adenomas showed a positivity for SSTRs (Table 2), although all of them showed expression of β -actin (data not shown). The subtypes SSTR1, SSTR2, SSTR3 and SSTR5 were equally expressed by about 50% (12/21, 11/21, 11/21 and 9/21 respectively) of the adenomas studied (Table 2). Conversely, SSTR4 was not expressed (Table 2). Interestingly, most of the NFPA (15/21) expressed more than one subtype of SSTR and two of them expressed four different SSTRs (SSTR1, 2, 3 and 5) (Table 2). The absence of genomic DNA contamination in the cDNA samples was assessed by the lack of any amplification product in a PCR reaction using non-reverse transcribed templates (data not shown).

In one case (P2) the PCR results obtained from the frozen sample were compared with the RT-PCR performed on RNA extracted from dispersed cultured cells.

The results showed the same pattern of expression between the two samples (only SSTR2 and SSTR5 were positive) confirming that after the dispersion and the culturing *in vitro* the expression of the receptors was unchanged.

Antiproliferative activity

Eleven of the adenomas were dispersed, cultured *in vitro* and tested for antiproliferative activity of somatostatin and its analog using the [^3H]thymidine uptake assay (P5 was analyzed only in calcium experiments). The SSTR subtypes expressed by the single adenomas tested *in vitro* are reported in Table 2. Fibroblast contamination was constantly extremely low since dispersed cells were kept in D-valine-containing medium that does not allow their proliferation, thus allowing a rather selective DNA synthesis analysis for adenoma cells (25, 26).

Basal activity was in most adenomas relatively low (250–524 c.p.m./100 000 cells) except in two cases that showed higher basal levels (1396 and 4299 c.p.m./100 000 cells). Preliminary experiments were conducted on two adenomas to identify possible *in vitro* stimulants of DNA synthesis. In particular we tested the effects of the direct activation of protein kinase C (PKC) by the tumor promoter phorbol myristate acetate (PMA) (100 nmol/l), and of protein kinase A (PKA) by the diterpene derivative forskolin (100 nmol/l). These preliminary experiments demonstrated that, while the activation of PKC resulted in a significant increase in DNA synthesis, the treatment with forskolin significantly reduced [^3H]thymidine incorporation (Fig. 1). Therefore, we evaluated the effects of lanreotide and somatostatin on NFPA in basal and PMA-stimulated conditions. Since the small amount of cells recovered from each tumor did not allow a complete pharmacological characterization (i.e. a dose-response or time-course study), we utilized a peptide concentration (1 $\mu\text{mol/l}$) that, in our hands, was able to induce a significant inhibition of cell proliferation *in vitro* (7).

The results obtained from six individual adenomas are shown in Fig. 2. Figure 3A reports the data from all the adenomas and Fig. 3B the average results obtained from all the 11 adenomas.

On the average, somatostatin treatment (1 $\mu\text{mol/l}$) did not result in a significant reduction of basal DNA synthesis although in two cases a statistically significant reduction occurred ($P_2 = -40\%$, $P_3 = -48\%$, $P < 0.05$) (Fig. 2, Fig. 3A and B).

Lanreotide (1 $\mu\text{mol/l}$) did not significantly modify basal [^3H]thymidine incorporation into DNA, in all the adenomas tested, including those with high basal activity (Fig. 2, Fig. 3A and B).

PMA (100 nmol/l) induced a significant increase in DNA synthesis in almost all the tumors studied with few exceptions (P7 and P9) (Fig. 2 and Fig. 3A). On average the PMA-stimulated [^3H]thymidine incorporation was about 50% higher than basal activity (Fig. 3A and B).

Figure 1 Effect of PMA (100 nmol/l) and forskolin (FSK) (100 nmol/l) on NIPA cell proliferation evaluated using the [3 H]thymidine uptake technique. Data, derived from two adenomas evaluated independently in quadruplicate, are expressed as c.p.m./100 000 plated cells $\pm$ S.E.M. * P <0.05 and ** P <0.01 vs basal value.

Somatostatin and lanreotide (1 μ mol/l) significantly inhibited the stimulatory activity of PMA reducing, on the average, the activity to basal levels (Fig. 3B).

Also in this parameter there was a certain variability among the different tumors. For instance the somatostatin effect was more pronounced compared with the lanreotide inhibition of DNA synthesis in two tumors (P2 and P3) and in another two (P1 and P7) only somatostatin was effective while lanreotide treatment was without effect. In other tumors lanreotide was more effective (P8, P9 and P12). In the remaining tumors there was no difference in the inhibitory activity of somatostatin and lanreotide (Fig. 2 and Fig. 3A).

Intracellular mechanisms

The activation of PTPs and the inhibition of the voltage-sensitive calcium channels represent two of the most relevant intracellular transducing pathways, activated by the SSTR, involved in the antiproliferative and endocrine activities of somatostatin (4).

The involvement of PTPs was evaluated indirectly by using the selective PTP inhibitor vanadate (33) in the proliferation assay in four adenomas (P6, P8, P9 and P11).

The average results obtained are reported in Fig. 4A (P6 and P11) and 4B (P8 and P9). Vanadate treatment (50 μ mol/l) resulted in a slight increase in the basal DNA synthesis activity in all the NFPA tested, as previously reported in some tumor cell lines (7, 28). The PTP inhibitor did not modify the PMA stimulation but significantly reduced the somatostatinergic inhibition of DNA synthesis in PMA-stimulated conditions in all the NFPA tested (Fig. 4A and B). The vanadate effect on lanreotide inhibition was more complex. Lanreotide, compared with somatostatin, caused a stronger inhibition of PMA-stimulated [3 H]thymidine incorporation. In

the presence of vanadate in two adenomas (P6 and P11, Fig. 4A) this inhibition was completely reversed, similar to what was observed after somatostatin treatment. Conversely, the other two adenomas (P8 and P9, Fig. 4B) showed no effect of vanadate on lanreotide inhibition of [3 H]thymidine uptake. Due to the scarcity of the surgical specimen, the somatostatin's effect on PTP activity, using the synthetic substrate p-Npp, was directly measured only in a single tumor (P12). Dispersed adenoma cells were treated with 1 μ mol/l somatostatin for 1 h. Total cell lysate (20 μ g) was then assayed for PTP activity. Somatostatin treatment induced a moderate but significant increase in the PTP activity (+33%) (Fig. 5) that paralleled the somatostatin inhibitory effects on DNA synthesis observed in the same adenoma (Fig. 3A).

The effect of somatostatin and lanreotide on K^+ -dependent [Ca^{2+}] $_i$ increase at the single cell level was evaluated in microfluorimetric studies using the fluorescent probe fura-2 in six adenomas (P1, P2, P5, P6, P7 and P11). In Fig. 6, the individual responses of three adenomas are shown, while Fig. 7 depicts the average response of all the adenomas tested. K^+ depolarization induced a rapid increase in [Ca^{2+}] $_i$ through the voltage-sensitive calcium channels, characterized by a spike followed by a plateau (Fig. 6). Both somatostatin and lanreotide pretreatments, although not changing basal [Ca^{2+}] $_i$, resulted in an average reduction of the spike of about 50% (Fig. 7) in all the adenomas tested, with a plateau phase constantly lower than in the control cells (Fig. 6).

Discussion

It was previously reported that most human NFPA express multiple SSTRs (19–21) but very few reports have shown the effects of somatostatin or its analogs on

Figure 2 Effect of somatostatin (SOM) (1 μ mol/l) and lanreotide (LAN) (1 μ mol/l) on basal and PMA (100 nmol/l)-stimulated DNA synthesis in cells of six NFPA. Experiments were performed independently in quadruplicate and the results are expressed as c.p.m./100 000 plated cells $\pm$ S.E.M., using the [³H]thymidine incorporation assay. The NFPA tested were: P1 (top left), P2 (middle left), P3 (bottom left), P6 (top right), P7 (middle right) and P8 (bottom right). * P <0.05, ** P <0.01 vs control value; ° P <0.05, °° P <0.01 vs pma value.

the proliferative activity of these tumors *in vitro* (26), and there are no previous published data analyzing the SSTR subtype and the possible intracellular mechanisms involved in such an effect.

In this study we analyzed SSTR expression in 21 NFPAAs. Only 2/21 (10%) did not express any SSTR mRNA. Among the other subtypes, SSTR4 was the only receptor not expressed, in agreement with previous reports (18, 20). Each of the SSTR1, 2, 3 and 5 subtypes

was expressed in similar percentages of the tumors (about 50%) and in most of the cases (70%) multiple subtypes were present in different combinations. The distribution of the SSTR subtypes in the different tumors is in agreement with previous reports (21) although in our samples we identified a higher presence of SSTR5. In our study, due to the paucity of the adenomatous tissue available, we performed only a qualitative analysis of the expression of single SSTRs. Further

Figure 3 (A) Summary of the results of the effects of somatostatin (som) (1 $\mu\text{mol/l}$) and lanreotide (lan) (1 $\mu\text{mol/l}$) on basal and PMA (pma) (100 nmol/l)-stimulated DNA synthesis in all the NFPAAs tested. Experiments were performed independently in quadruplicate and the results are expressed as the percentage of the changes in [^3H]thymidine incorporation vs the control (con) value. (B) Average of the results of the effects of somatostatin (som) and lanreotide (lan) on basal and PMA-stimulated DNA synthesis, reported in panel (A). ** $P < 0.01$ vs control value; °° $P < 0.01$ vs PMA value.

Figure 4 Effect of vanadate (50 $\mu\text{mol/l}$) pretreatment on somatostatin (SOM) (1 $\mu\text{mol/l}$) and lanreotide (LAN) (1 $\mu\text{mol/l}$) inhibition of PMA (100 nmol/l)-stimulated DNA synthesis. Control=open bars; vanadate=hatched bars. (A) Average of the data obtained from P6 and P11 NFPA, expressed as a percentage of basal activity. (B) Average of the data obtained from P8 and P9 NFPA, expressed as a percentage of basal activity. ** $P < 0.01$ vs respective basal value; ° $P < 0.05$ and °° $P < 0.01$ vs PMA value; + $P < 0.05$ vs PMA + LAN control value; * $P < 0.05$ vs PMA + SOM control value.

studies will be necessary to identify the different levels of expression of the SSTRs, with the possible use of receptor-selective somatostatin analogs.

Although a direct antiproliferative activity of somatostatin on somatotrophic human pituitary adenomas was suggested by experimental (26) and clinical studies that showed a tumor shrinkage during the pre-operative treatment of these patients with somatostatin analogs

(34), limited evidence is available on NFPA. In this study we cultured adenomatous cells *in vitro* and evaluated the proliferative activity by means of a [^3H]thymidine uptake assay. As previously reported (35), *in vitro* basal DNA synthesis activity varied among the different tumors. The PKC enzyme family regulates many cellular processes including cell proliferation and differentiation. In our experimental conditions the

Figure 5 Somatostatin stimulates PTP activity in cell lysate from dispersed pituitary adenoma cells (P12). Cells were pre-treated for 1 h with somatostatin (1 $\mu\text{mol/l}$) and then harvested for the enzymatic assay. PTP activity was evaluated using the synthetic substrate p-Npp, in the presence of the inhibitor of serine/threonine phosphatases microcystin-leucine-arginine. * $P < 0.05$ vs control value.

Figure 6 Effect of somatostatin ($10 \mu\text{mol/l}$) and lanreotide ($10 \mu\text{mol/l}$) on KCl (40 mmol/l)-induced activation of voltage-sensitive calcium channels at the single cell level in cells derived from three individual NFPAs. The traces are the average of multiple cells. $\circ$ Control; $\blacksquare$ somatostatin (SOM); $\times$ lanreotide (LAN). The NFPAs reported in the figure were: P1 (top), P5 (middle), P6 (bottom). The number of cells analyzed for each trace was: P1: control $n=19$, SOM $n=25$; P5: control $n=22$, SOM $n=13$, LAN $n=11$; P6: control $n=7$, LAN $n=5$. The arrows indicate the time of administration of the test substances.

Figure 7 Effect of somatostatin (10 μ mol/l) and lanreotide (10 μ mol/l) on KCl (40 mmol/l)-induced activation of voltage-sensitive calcium channels at the single cell level. Data represent the average of the results obtained from six individual NFPA cells. The figure shows the fold increase in $[Ca^{2+}]_i$ during the spike phase induced by KCl depolarization, in control cells or in cells pre-treated with somatostatin (Som) or lanreotide (Lan). * $P < 0.05$ vs control value.

activation of PKC, but not of PKA, was able to induce DNA synthesis in dispersed human NFPA cells. These data are in agreement with the observation by Alvaro *et al.* (36), who reported an increase in PKC activity in NFPA cells compared with normal pituitary and higher expression of PKC in invasive adenomas compared with non-invasive. Moreover, in pituitary adenoma cell lines, the PKC inhibitor hypericin induced growth arrest and apoptosis (37). Conversely, the forskolin-dependent activation of the cAMP/PKA system was not only unable to stimulate DNA synthesis in the primary cultures of NFPA cells but resulted in a significant inhibition of [3H]thymidine incorporation. Indeed, the

proposed involvement of cAMP/PKA mechanisms in the pathogenesis of somatotrophic pituitary adenomas, through the *gsp* mutation in the $G\alpha$ -subunit of the protein G (38), was not observed in NFPA cells, which expressed the *gsp* oncogene only in a small percentage of cases (39, 40).

Both somatostatin and lanreotide were able to significantly inhibit the proliferative activity of PMA, although some difference in the response among the different tumors was observed. The results obtained using a somatostatin analog that selectively binds the SSTR2 and SSTR5 subtypes (41) strongly suggest the involvement of these receptors in the antiproliferative activity of somatostatin. However, the low amount of available cultured cells from each adenoma made it impossible to perform dose-response experiments that could have allowed, at least for the lanreotide experiments, a pharmacological identification of possible SSTRs involved in the antiproliferative activity. The observation that in some tumors somatostatin was more effective than lanreotide, although the latter peptide was much more long-lasting, and that in others only somatostatin was able to inhibit cell proliferation, indicates that other receptor subtypes may also be involved in these effects. The different expression of the SSTR subtypes in the adenomas that we studied does not allow a correlation between single receptor subtypes and the somatostatinergic inhibition of cell proliferation. Thus, it is likely that more SSTRs are able to induce such an effect and that when multiple subtypes are expressed they may co-operate to induce growth arrest. Activation of PTP represents one of the major intracellular signal pathways responsible for the antiproliferative effects of somatostatin (7, 15). Here we report, for the first time in primary cultures derived from human tumor tissues, that also in NFPA cells SSTRs may be coupled to this second messenger system. However, since a direct analysis of the somatostatin activation of PTP was performed in only one adenoma, further studies will be necessary to confirm this observation.

Table 2 Non-functioning adenomas: SSTR expression.

	SSTR Expression				
	1	2	3	4	5
P 1	N	N	N	N	N
P 2	—	+	—	—	—
P 3	N	N	N	N	N
P 4	+	+	+	—	—
P 5	—	—	—	—	+
P 6	+	+	—	—	—
P 7	+	+	—	—	—
P 8	+	+	+	—	+
P 9	+	+	+	—	+
P10	+	+	+	—	—
P11	—	+	+	—	+
P12	—	+	+	—	—
P13	—	—	+	—	—
P14	+	—	+	—	—
P15	+	—	+	—	+
P16	+	—	—	—	+
P17	—	—	+	—	+
P18	—	—	+	—	+
P19	+	—	—	—	—
P20	—	—	—	—	—
P21	—	+	—	—	—
P22	+	—	—	—	+
P23	+	+	+	—	—

N = not done.

Interestingly, SSTRs appear also to be coupled to an inactivation of voltage-sensitive calcium channels in NFPA cells, as previously reported in other cell types (42). Calcium influx, beside its well-known secretagogue activity, was reported to control cell proliferation through the regulation of kinase cascade and gene transcription (43, 44). Thus, in our experimental model at least two different second messenger systems seem to be affected by SSTR activation and are likely involved in the antiproliferative activity of the peptide.

As regards the antiproliferative effects, the SSTR subtypes specifically coupled with the single second messengers were not identifiable from our data, since the adenomas studied, which were all responsive to somatostatin and lanreotide, showed different patterns of receptor expression. However, a participation of SSTR2 and SSTR5 in the inhibition of calcium fluxes can be supported by the results obtained with lanreotide. Indeed, although multiple SSTRs, including SSTR1, SSTR2 and SSTR5 have been previously reported to modulate calcium fluxes (45, 46), it was reported that only SSTR2 and SSTR5 are involved in the somatostatin-dependent inhibition of α -subunit secretion (19), a function strictly related to $[Ca^{2+}]_i$ regulation. In contrast to previous reports, in our study we did not observe a somatostatin-dependent inhibition of basal $[Ca^{2+}]_i$ (47, 48). However, in our experimental conditions basal $[Ca^{2+}]_i$ was lower (between 10 and 50 nmol/l) than that previously reported in normal rat somatotrophs (47) or human growth hormone-secreting adenoma cells (48). Thus in our experimental conditions a further reduction in $[Ca^{2+}]_i$ may be undetectable because it is below the threshold of detection of the microfluorimeter.

Vanadate pretreatment reversed the lanreotide inhibition of DNA synthesis only in two of the four adenomas tested, although there were no significant differences in the expression of SSTRs (for instance SSTR2 was expressed in all the adenomas analyzed). These results, confirming that SSTR2 may be coupled to PTP to inhibit cell proliferation (12, 49), suggest that the capability of activating this transduction system in tumor tissues may depend, not only on the receptor subtype present on the cell membrane, but also on the expression of specific intracellular effector molecules, such as specific PTPs activated by SSTR2. Indeed, with the two adenomas where the effect of lanreotide was not reversed by vanadate, the effect of somatostatin was blocked. These tumors expressed SSTR1, a receptor subtype reported to be coupled to PTP (12, 13), but which does not bind lanreotide. Moreover, we recently reported in transformed rat thyroid cell lines the loss of expression of a specific PTP, named PTP η that correlated with the lack of effects of somatostatin on cell proliferation (7, 50), suggesting the requirement of both receptor subtype and specific effector molecules for the somatostatin antiproliferative effects. Moreover, it is noteworthy that, at least in the NFPA cells we studied, the

lack of coupling of SSTRs with PTP did not result in a loss of the antiproliferative effects of lanreotide. Since these adenomas both expressed SSTR5 it is possible that in these tumors the proliferative activity was under the control of the lanreotide inhibitory effects through this receptor subtype acting on the phospholipase C activity, as previously reported (51).

In conclusion our data show that a high percentage of human NFPA cells express multiple SSTRs. The activation of these receptors *in vitro* by somatostatin or lanreotide results in a reduction of the proliferation of the adenomatous cells. This effect is correlated to both an increase in PTP activity and an inhibition of voltage-dependent calcium channel activity that may represent possible intracellular effectors of the antiproliferative signals started by SSTRs activation.

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