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Mechanisms of Somatostatin Action in RINm5F Cells in Culture: Preliminary Evidence for Possible Altered G Protein Function[1]

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Octreotide (SMS), a somatostatin analogue, is an established antigrowth peptide, but it does not effectively inhibit the growth of insulinoma cells. In order to study the mechanisms that underlie this apparent lack of an antiproliferative effect on insulinoma tumor cells we established the rat insulinoma cell line, RINm5F, in culture. Cells in culture were tested by incubation in media with and without SMS. To study tritiated [^{3}H]-thymidine incorporation into extracted DNA (TTID), 2 μCi/well of ^{3}H was added for 24 hr, and cells were harvested and assayed for TTID (cpm/μg DNA). Insulin (IRI) and intracellular cAMP (cAMP$_i$) were measured by RIA. To study the effects of SMS on insulin secretion, conditioned media were sampled after 24 hr. To study the effects of cAMP$_i$, conditioned medium was used to extract cAMP$_i$ following incubation with SMS for 15 min. Increasing concentrations of SMS had no significant effect on TTID in the presence of 1% FBS. Trypan blue exclusion tests showed >90% viable cells throughout all stages of these experiments. There were no significant differences in cell numbers and protein content in the presence of SMS. There was a significant decrease in the secretion of insulin and intracellular cAMP levels in response to 50 n*M* SMS. However, SMS significantly inhibited TTID in RINm5F cells following a 4-hr pretreatment with pertussis toxin (PT) (23553 ± 1747 vs 20635 [cpm/μg DNA] ± 1983 [SEM], $P < 0.01$). We conclude that the inhibition of insulin secretion by SMS is associated with an attenuation of cAMP formation. However, the effects of SMS on cell proliferation appear to be distinct and more complex. The unexpected ability of PT to induce the inhibition of TTID by SMS suggests that SMS mediates, at least some of, its effects on cell proliferation in RINm5F cells via a possibly altered G protein or related signal transducing mechanism. Further study of the signal transduction system in RINm5F cells may elucidate the mechanisms of tumor growth in insulinoma.

INTRODUCTION

Somatostatin, a cyclic tetradecapeptide initially isolated from ovine hypothalamus as an inhibitor of growth hormone release, has been localized in many other tissues [1]. It is a well-established antisecretory and antiproliferative peptide. Sandostatin (octreotide, SMS 201-995, Sandoz Pharmaceutical Co., East Hanover, NJ) is a long-acting, octapeptide analogue of somatostatin. It, similarly, has demonstrated antiproliferative effects (both *in vivo* and *in vitro*) on a variety of solid tumors including breast [2], prostate [3, 4], colon [5], pancreatic [6], and small-cell lung carcinoma [7], as well as, other clonal tumor cells [8, 9]. Also, it has been shown to inhibit peptide secretion in patients with functioning endocrine tumors [10–12]. However, octreotide (SMS) does not effectively inhibit the growth of cells in insulinomas, despite it being a well-established antigrowth peptide.

Transmembrane signal (hormone, growth factor, etc.) receptors are associated with a class of proteins bound to the cytoplasmic side of the cell membrane which bind to guanine nucleotides such as guanosine triphosphate (GTP) with high affinity [13]. These membrane-bound cytoplasmic guanine nucleotide-binding proteins, called G proteins [14, 15], are linked to protein synthesis [13, 16] and are believed to play a role in controlling cell proliferation and growth [17].

Somatostatin is known to act via an inhibitory guanine nucleotide binding protein (G_i) in many cell systems [18–25]. The absence of any demonstrable somatostatin-induced antiproliferative effect on insulinoma cells suggests that there may be either an altered or a different guanine nucleotide subunit protein, or a related transduction mechanism in insulinomas. To study this further, we established the rat insulinoma cell line,

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RINm5F, in culture to use as a model for human insulinoma. The RINm5F cell line was originally established from an X-ray induced rat insulinoma [26] and has been shown to store and release immunoreactive insulin [27]. The release of insulin from RINm5F cells is stimulated by a variety of secretagogues (amino acids and triose glyceraldehyde), but not by glucose [28, 29].

METHODS

RINm5F cells were maintained in 75-cm^2 tissue culture flasks at 37°C in 5% CO_2 in Roswell Park Memorial Institute 1640 culture media (RPMI-1640) supplemented with 5% heat-inactivated fetal bovine serum, 2 m*M* L-glutamine, 1% penicillin, and 1% streptomycin and were allowed to grow to confluence before beginning each experimental protocol. In preparation for each experiment, cells were detached using 0.05% trypsin–0.53% m*M* NaEDTA in Hank's balanced salt solution and subsequently were plated onto 5 wells in 6-well tissue culture plates and grown in 2 ml medium supplemented with 1% fetal bovine serum × 48 hr. This was followed by 2 ml of standard media (RPMI-1640 with 2 m*M* L-glutamine, 1% penicillin, and 1% streptomycin) supplemented with 0.5% fetal bovine serum × 24 hr. The medium was aspirated and 2 ml fresh medium was added at the start of all test conditions. The viability of cells was tested by trypan blue exclusion tests. These tests showed >90% viable cells throughout all stages of these experiments.

Treatment with Pertussis Toxin

RINm5F cells were pretreated with pertussis toxin (PT) (100 ng/ml) for 4 hr. Studies in which RINm5F cells were preincubated with concentrations of PT ranging from 50 to 400 ng/ml produced results that were similar to those obtained with 100 ng/ml for a 4-hr pretreatment. The wells containing PT were aspirated, replaced with 2 ml medium, and the respective additive dosages of octreotide were used for each test condition.

Treatment with Octreotide (SMS 201-995)

Cells were incubated with octreotide (50, 500, or 5000 n*M*) × 16 hr prior to a 24-hr pulse with tritiated thymidine. Previous experiments have demonstrated that throughout a range of 5×10^{-4} *M* to 10^{-15} *M* octreotide 50 and 500 n*M* most consistently produced the maximum responses in tritiated thymidine incorporation into extracted DNA [30].

Tritiated Thymidine Uptake

Following a 16-hr incubation, 2 μCi/well [methyl-^{3}H]-thymidine (Amersham) was added to cells. RINm5F cells were incubated for 24 hr, then washed with cold phosphate-buffered saline. Two milliliters of 6% trichloroacetic acid was added to each well, and cells were scraped and rinsed from tissue culture plates. Cell fragments were centrifuged ×2 at 1800 rpm for 20 min. The supernatant was used to measure the protein content and counts in a scintillation counter. The cell pellet was treated with 3% perchloric acid (PCA), heated at 95°C for 10 min, iced for 20 min, and then centrifuged at 2900 rpm for 10 min. Supernatant was used to measure DNA by direct assay.

DNA Assay

Two hundred and fifty microliters of 3% perchloric acid was added to assay tubes. DNA standard was prepared using 0.3 mg/ml DNA (calf thymus, Sigma) in 5 m*M* NaOH diluted 1:1 in 1 *N* perchloric acid, heated to 70°C for 15 min.

A DNA standard curve was used to estimate unknown values. One milliliter of diphenylamine reagent was added to a total volume of 500-μl samples and incubated at 30°C for 6–24 hr. Samples were read at 600 nm on a spectrophotometer (Hitachi, Model 100-40). Results for tritiated thymidine incorporation into extracted DNA were calculated and expressed as counts per minute per microgram of DNA.

Insulin

To study the effects of octreotide on insulin secretion, conditioned media were sampled after 24 hr with and without octreotide. Replicates ×5 were used in each experiment. Results are expressed as mean ± SEM ($N \geq 3$).

Immunoreactive insulin was measured by using a double-antibody radioimmunoassay with an ^{125}I-porcine insulin tracer, a rat insulin standard (Novo), a guinea pig anti-rat insulin first antibody (Linco Research), and a sheep anti-guinea pig gamma globulin-PEG second antibody. Limit of sensitivity for the assay was 3 μU/ml. Interassay and intraassay coefficients of variability were 3.2 and 1.4%, respectively.

Cyclic Adenosine Monophosphate (cAMP)

Intracellular cAMP was measured following incubation with octreotide for 15 min. Cold 6% TCA (1 ml) was added to wells, and cells were scraped off using a rubber policeman. Fragments and TCA were collected and placed into tubes (5 wells/tube) and centrifuged at 1800 rpm for 20 min. The supernatant was washed in 4 × 4 ml with diethyl ether (saturated with water), and 200 ml from the aqueous layer was assayed for immunoreactive cAMP.

A double-antibody radioimmunoassay using an ^{125}I tracer (New England Nuclear), a rabbit anti-cAMP first antibody (Vaitukatis, Boston City Hospital), and a sheep anti-rabbit IgG second antibody was applied. The sample and standards are acetylated prior to assaying

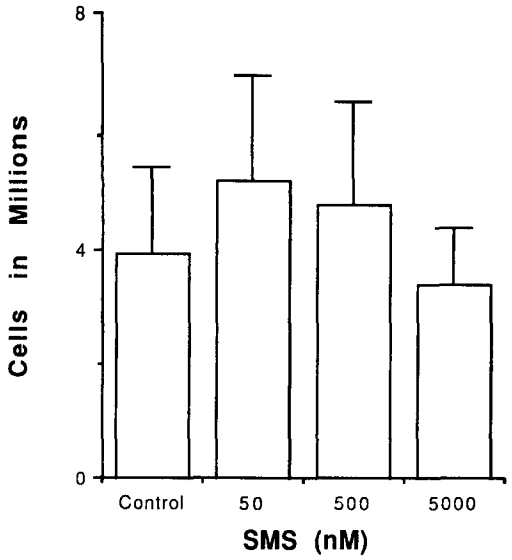

FIG. 1. The response of cell number in RINm5F cells to various doses of octreotide (SMS). RINm5F cells (10^6) were plated and grown as described under Methods. The media was then aspirated. Cells were then incubated in test media containing 1% fetal bovine serum for 24 hr. Cells were then scraped and viable cells as determined by trypan blue exclusion rest were counted using hemocytometer. The data are representative of five experiments. The vertical lines represent ± SEM. For experimental details, see Methods.

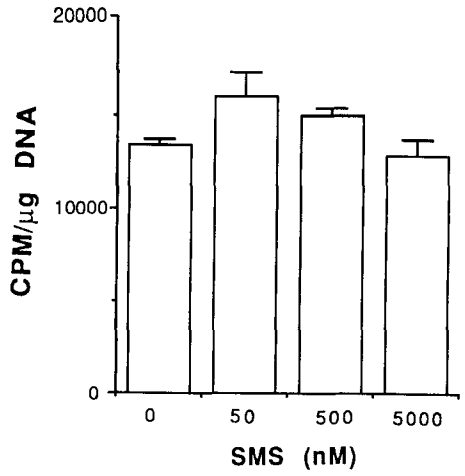

FIG. 2. The effect of various doses of octreotide (SMS) on tritiated thymidine incorporation into extracted DNA from RINm5F cells as indicated by cpm/μg DNA on the ordinate-axis. The data are representative of three or more experiments. The vertical lines represent ± SEM. For experimental details, see Methods, and Figure 1.

for greater sensitivity. Limit of sensitivity is 17.8 fmole/ml. Interassay and intraassay coefficients of variability are 5.4 and 1.5%, respectively.

Statistics

Significant differences between treatment groups were tested for using the Wilcoxon rank sign test and the Student *t* test. A *P* value ≤ 0.05 was the criterion used to identify a significant difference.

RESULTS

Effect of Octreotide on Cell Number and Tritiated Thymidine Incorporation

A number of studies have shown that somatostatin or one of its analogues (SMS) inhibit cell proliferation [7–9, 23]. In contrast to these studies, there was no significant inhibition in cell numbers with increasing concentrations of octreotide (50–5000 n*M*) in the RINm5F cell (shown in Fig. 1). Similarly, there were no significant changes in protein content observed in RINm5F cells incubated in the presence of the same range of dosages of octreotide (Table 1).

To further study the observed lack of effect of octreotide on RINm5F cell number, we examined the effect of octreotide on tritiated thymidine incorporation into the extracted DNA of these cells. Increasing concentrations of octreotide (50–5000 n*M*) had no significant effect on the extent of tritiated thymidine incorporation into extracted DNA (TTID) in RINm5F cells incubated in the presence of standard media supplemented with 1% fetal bovine serum (shown in Fig. 2).

Effect of Octreotide on Insulin Secretion and Intracellular Cyclic AMP

Insulin secretion (IRI) and intracellular cyclic AMP levels were significantly decreased in RINm5F cells

TABLE 1

Effect of Octreotide (SMS) on Protein Content

Test conditions (Reagents)	Protein content (μg) ($n = 5$)
Control	108 ± 5
50 n*M* SMS	102 ± 5
500 n*M* SMS	110 ± 3
5000 n*M* SMS	120 ± 7

Note. Protein content was measured by the Lowry method [42]. See Methods for details. Data are expressed as the mean ± SEM from triplicate determinations from five experiments.

TABLE 2

Effect of Octreotide (SMS) on Insulin Secretion and Intracellular Cyclic AMP Formation

Assay	Control	SMS 50 n*M*
Insulin (μU/ml)	159 ± 18	129 ± 15*
Cyclic AMP (fmole/ml)	1233 ± 213	1053 ± 180*

Note. Insulin and cyclic AMP were measured by insulin-specific and cyclic AMP-specific RIAs, respectively. For details see Methods. Data are expressed as the mean ± SEM of 9–12 determinations from three or more experiments.

* $P < 0.05$ from control by Wilcoxon signed rank test.

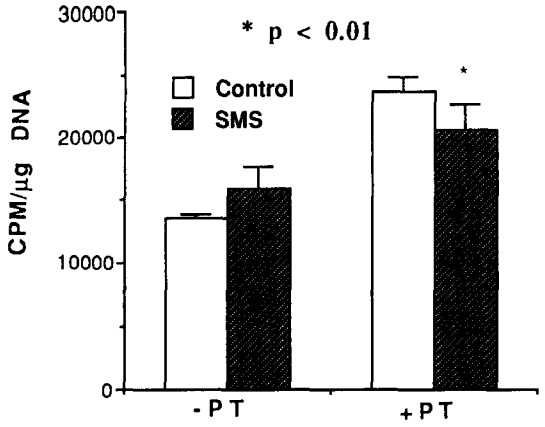

FIG. 3. The response of tritiated thymidine incorporation into extracted DNA (cpm/μg DNA) in RINm5F cells incubated in standard media + 1% fetal bovine serum either alone (control media/control) or in control media supplemented with 50 n*M* octreotide (SMS). Both conditions were subjected to either a 4-hr pretreatment with pertussis toxin (100 ng/mL) [+PT] or a 4-hr pretreatment without pertussis toxin [−PT]. The data are representative of three or more experiments. The vertical lines represent ± SEM. For experimental details, see Methods.

grown under previously described experimental conditions in response to SMS (50 n*M*) (see Table 2).

Effect of Octreotide on TTID after Pretreatment with Pertussis Toxin

Pretreatment with pertussis toxin is known to disrupt the inhibitory alpha subunit of the guanine nucleotide-binding regulatory component (G_i) by ADP-ribosylation and thereby block the inhibitory effect of SMS on the adenylate cyclase system. In these experiments, octreotide alone did not inhibit tritiated thymidine incorporation into extracted DNA; however, octreotide significantly inhibited tritiated thymidine incorporation into extracted DNA in RINm5F cells pretreated with pertussis toxin (100 ng/ml/4 hr) (23553 ± 1747 vs 20635 [cpm/μg DNA] ± 1983 [SEM], $P < 0.01$) (shown in Fig. 3). Similar results were observed even after pretreatment with 400 ng/ml/4 hr of pertussis toxin (data not shown). In addition, octreotide also inhibited insulin secretion into condition media from 47 ± 4.0 to 40 ± 4.2 (μU/ml ± SEM) in RINm5F cells pretreated with 50 ng/ml of pertussis toxin.

DISCUSSION

Somatostatin or its analogue is known to be a potent antisecretory and antigrowth peptide [31]. However, the exact mechanism(s) for its antigrowth effects are unknown. There are several fundamental mechanisms by which cell growth in endocrine cells may become abnormal. These mechanisms include abnormalities of growth factor production, abnormalities of growth factor receptors, reduced production of growth-inhibitory regulatory factors, or disturbances of post-receptor signal transduction [32]. Somatostatin may play a role in one or more of these fundamental mechanisms controlling abnormal (neoplastic) growth in any given cell.

Somatostatin was initially isolated based on its ability to inhibit growth hormone secretion from rat anterior pituitary cells in culture [1]. Subsequently, it has been shown to inhibit the secretion of several other hormones by pituitary cells in culture [33]. Furthermore, it has been shown that somatostatin inhibits such hormonal secretion by attenuating the formation of intracellular cyclic AMP [34, 35]. Our data demonstrating that octreotide inhibits insulin secretion and intracellular cyclic AMP levels in RINm5F cells are consistent with these reported observations.

Human studies using octreotide in the treatment of patients with malignant insulinomas have shown that octreotide does not reduce the size or inhibit the growth of the tumor, but that it does reduce the levels of circulating insulin (IRI) in such patients [36–38]. Our *in vitro* studies using a rat insulinoma cell line, RINm5F, also showed that increasing concentrations of octreotide did not reduce or inhibit the proliferation of cells, but it did reduce the levels of immunoreactive insulin secreted into the conditioned media (see Figs. 1 and 2, Table 2). In addition, we did not observe any significant changes in the protein content of these cells following treatment with octreotide. The parallel patterns of response of RINm5F cells in culture to those observed for human insulinomas suggest that our experimental model using RINm5F cells *in vitro* is a functional and useful model with which to investigate the mechanisms of peptide action in insulinomas.

Pertussis toxin is known to ADP-ribosylate the inhibitory alpha subunit of the membrane-bound guanine nucleotide binding protein, G_i. Somatostatin is known to mediate several of its postreceptor effects via the membrane-bound inhibitory subunit of guanine nucleotide binding protein (G_i). Furthermore, it has been shown that the inhibitory effects of somatostatin can be blocked by pretreatment with pertussis toxin [18, 23, 24, 39].

In these experiments, pertussis toxin pretreatment followed by treatment with octreotide resulted in a significant inhibition of tritiated thymidine incorporation into the extracted DNA of RINm5F cells. The mechanism for this net inhibitory effect of octreotide in RINm5F cells only after pretreatment with pertussis toxin is unclear. It is possible that octreotide exerts both stimulatory and inhibitory effects on cell proliferation in RINm5F cells and that the stimulatory effects are pertussis toxin-sensitive. Alternatively, G_i in the RINm5F cell may be altered, expressed differently, or associated with an abnormal or a different signal transducting mechanism in these cells. Consequently, pertussis toxin may exert a net activation of G_i in the RINm5F cell.

Alteration of G_i in disease states is not without prece-

dent; for example, it has been shown that in patients with non-insulin-dependent diabetes, G_i expression is altered [40]. The fact that pretreatment of RINm5F cells with pertussis toxin did not significantly alter (block) the inhibitory action of octreotide on insulin secretion (data described under Results) further supports the possibility that G_i may be altered or expressed differently in RINm5F cells. The observation that pretreatment of RINm5F cells with pertussis toxin significantly altered the response of tritiated thymidine incorporation into DNA in these experiments further highlights the probability that the mechanisms mediating peptide secretion may be distinct from those mechanisms mediating cell proliferation. Recently reported findings on the existence of several distinct clones of somatostatin receptors in several tissues with apparent distinct cell effectors are consistent with this probability [41].

In conclusion, we have established a viable model to study the mechanisms of action of somatostatin in insulinomas. We have shown that there appears to be two distinct mechanisms of action of somatostatin in RINm5F cells. It appears that octreotide exerts its inhibitory action on peptide secretion by inhibiting or attenuating cyclic AMP formation, whereas its antiproliferative action appears to be related to either the net activation of G_i, or via an altered or different G protein mechanism. Peptide receptors regulate the adenylyl cyclase system and related cell effector systems via G proteins, as well as second messenger systems that include cyclic AMP. Further study of the signal transduction system in RINm5F cells may elucidate the apparently distinct mechanisms of peptide secretion and tumor cell proliferation in insulinoma.

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