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Up-regulation of Somatostatin Receptors by Epidermal Growth Factor and Gastrin in Pancreatic Cancer Cells

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SUMMARY

Interactions between growth factor receptor systems may be important in the regulation of cell growth. The proliferation of pancreatic tumor AR42J cells has been shown to be stimulated by Epidermal growth factor (EGF) and gastrin and inhibited by somatostatin. To analyze the interaction between these different peptides, we explored the influence of EGF and gastrin on the somatostatin receptors. Treatment of AR42J cells with 10 nM EGF or gastrin for 24 hr increased specific binding of [^{125}I]Tyr^{3}SMS to 131 and 147% of that in control cells, respectively. The effect of peptides on [^{125}I]Tyr^{3}SMS binding was time- and dose-dependent, with half-maximal effect at 0.2 ± 0.03 nM EGF and 0.3 ± 0.15 nM gastrin. Scatchard plots revealed an increase in somatostatin receptor number of 27 and 80% after 48 hr of treatment with EGF and gastrin, respectively, without any change in receptor affinity. The increase in somatostatin receptor density was accompanied by the enhancement of biological responses to somatostatin. In cells pretreated with EGF or gastrin, the potency of somatostatin for inhibiting vasoactive intestinal peptide-stimulated cAMP content was increased 2-fold as that of somatostatin analog, SMS, for inhibiting cell proliferation. Furthermore, the efficiency of SMS as antiproliferative agent was greatly increased. Vasoactive intestinal peptide or forskolin did not modify [^{125}I]Tyr^{3}SMS binding of control or treated cells. The phorbol ester 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) did not affect [^{125}I]Tyr^{3}SMS binding. On the other hand, cycloheximide completely blocked the increase in [^{125}I]Tyr^{3}SMS binding induced by EGF and gastrin. Analysis of mRNA expression of the SSTR1, 2, 3 somatostatin receptor subtypes demonstrated that in AR42J cells SSTR1 and SSTR3 mRNAs were detected at very low levels, whereas the steady-state level of SSTR2 mRNA was high. EGF and gastrin enhanced the steady-state level of SSTR2 mRNA. The increase was time dependent and reached 72 and 200% after 24 hr of treatment with EGF and gastrin, respectively. EGF and gastrin also enhanced the level of SSTR3 mRNA by 300 and 290%, respectively, after 24 hr of treatment. In contrast, these agents had no effect on SSTR1 mRNA levels. We conclude that EGF and gastrin up-regulate functional somatostatin receptors through a protein kinase A- and protein kinase C-independent pathways. This effect requires protein synthesis and is mediated, at least in part, by the increase of SSTR2 mRNA levels and, to a lower extent, by that of SSTR3.

Somatostatin is a widely distributed polypeptide hormone that has been shown to play an important regulatory role on multiple target organs including brain, gastrointestinal tract, and pancreas. It inhibits a variety of biological processes, such as neurotransmission and hormone secretions (1). In addition, somatostatin acts as a negative regulator of normal and tumor cell growth and modulates growth factor or hormone-induced cell proliferation (2–5).

Somatostatin exerts its effects by interacting with specific receptors that have been characterized in various tissues (6–8) and shown to be coupled to different signal transduction pathways including adenylate cyclase, K^+ channels, voltage-dependent Ca^{2+} channels, and phosphatases (9–11). The recent cloning of somatostatin receptors demonstrates that they belong to the super family of G protein-coupled receptors with seven α-helical transmembrane segments (12).

Cell surface receptor regulation by heterologous ligands (transmodulation) is known to be an important mechanism in the physiological response to a given factor. For instance, modulation of a growth factor receptor by other structurally unrelated growth mediators is an important step in the control of cell proliferation and differentiation (13, 14). Somatostatin receptors are targets for heterologous regulation by steroids hormones. Glucocorticoid treatment decreases whereas β-estradiol increases the number of somatostatin receptors (15, 16). The observation that somatostatin antagonizes the mitogenic effect of peptides and growth factors (3–5) raises the possibility that the mitogenic agents may regulate somatostatin receptors.

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ABBREVIATIONS: bp, base pair(s); PCR, polymerase chain reaction; EGF, epidermal growth factor; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate; DMEM, Dulbecco's modified Eagle's medium; VIP, vasoactive intestinal peptide.

In the present study, we have investigated the somatostatin receptor modulation by two mitogenic factors, EGF and gastrin (5, 17), in AR42J rat pancreatic tumor acinar cell line. This cell line has been shown to contain functional somatostatin receptors (18) and treatment of cells with SMS 201–995 (SMS), a synthetic somatostatin analog, inhibited both serum- and growth factor-induced AR42J cell proliferation (5, 19). We present evidence that in this tumor cell line, both EGF and gastrin increased functional somatostatin receptors. This regulatory effect was independent of cAMP and protein kinase C pathways, required new protein synthesis, and could be related to increase in SSTR2 and SSTR3 somatostatin receptor subtype mRNA levels.

Experimental Procedures

Materials. Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were from Life Technologies, Inc. SMS [DPhe-Cys-Phe-DTrp-Lys-Thr-Cys-Thr(ol)] and Tyr^{3}SMS were a kind gift of Drs. A. Harris and P. Marbach (Sandoz, Basel, Switzerland). EGF (receptor grade) was purchased from Collaborative Research. Gastrin was from Neosystem (Strasbourg, France). CR 1409 was provided by Dr. L. Rovatti (Rotta Research Laboratory, Milan, Italy). ^{125}I (10 mCi/mmol) and [α-^{33}P]dCTP (1650 Ci/mmol) from Amersham International (Les Ulis, France). 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) and forskolin were purchased from Sigma. Reverse transcriptase MMLV was from Life Technologies, Inc., *Taq* polymerase was from Beckman, and pGEM DNA markers were from Promega. Primers were synthesized by Bioprobe systems. Geneclean II kit was from BIO 101 Inc. (La Jolla, CA), and dsDNA Cycle Sequencing System kit was from Life Technologies, Inc.

Cell culture. AR42J cells were grown in DMEM containing 10% fetal calf serum. Cells were routinely plated into 175 cm^2 flasks. Growth medium was changed every second day. Confluent cells were trypsinized and seeded in DMEM containing fetal calf serum at 25,000 cells/cm^2 in 35-mm dishes for binding experiments and growth assays or in 16-mm multiwell plates for cAMP assays. After 14 hr for cell attachment, the medium was changed to serum-free DMEM, and cells were exposed to different factors for specified times. Agents were added each day. Cell number was determined with a Coulter model 2 M counter (Coulter Electronics), after treatment of cells with 0.05% trypsin and 0.02% EDTA.

Binding studies. For somatostatin binding assays, Tyr^{3}SMS was radioiodinated and purified by high performance liquid chromatography as previously described (5). After incubation of cells with the different factors, cells were washed twice with phosphate buffer, scraped, and suspended at 10^5 cells/0.25 ml in Krebs-HEPES medium containing bacitracin (0.5 mM), soybean trypsin inhibitor (0.3 mg/ml), bovine serum albumin (0.2%), pH 7.35 (standard buffer). Binding reaction was carried out for 75 min at 25°, in the presence of 100 pM [^{125}I]Tyr^{3}SMS. Nonspecific binding was determined by adding 1 μM of unlabeled SMS and was about 10% of total binding. Separation of bound and free ligand was obtained by filtration on GF/C filters (Whatman) and by washing three times with cold standard buffer. Each data point in a given experiment represented a triplicate determination. Data analysis of competition isotherms were carried out on an IBM PC computer with a curve-fitting computer program (Ligand program, Biomedical Computing Technology Information Center, Nashville, TN), which is designed to estimate the equilibrium dissociation constant (K_d) and the receptor binding capacity (B_{max}).

Measurement of cAMP content in AR42J cells. AR42J cells were washed twice and incubated for 30 min at 25° in 1 ml of standard buffer. Then, the medium was removed, and cells were incubated in 300 μl of peptide-containing medium supplemented with 200 μM isobutylmethylxanthine for a further 30 min at 25°. The reaction was terminated by adding 600 μl of ice-cold ethanol 95°. Cells were kept at 4° for 4 hr, scraped, and rinsed with 300 μl of ice-cold ethanol 95°. Cells were then centrifuged at 3,000 × *g* for 15 min, and duplicate 10-μl samples of supernatant were assayed for cAMP by a radioassay kit (NEN/Dupont de Nemours, France).

Reverse transcription polymerase chain reaction for detection of SSTR1, SSTR2, and SSTR3 somatostatin receptor subtype mRNAs. Total RNA was extracted by a procedure derived from that of Chomczynski and Sacchi (20). Cells were lyzed in RNazol B for 10 min at 4°, and RNA was extracted with chloroform for 5 min at 4°. After centrifugation at 12,000 × *g* for 30 min at 4°, the extracted RNA was precipitated with isopropanol and washed with 75% ethanol. The RNA pellet was resuspended in diethylpyrocarbonate-treated water. The integrity of mRNA was controlled by analyzing rRNA contents on formaldehyde-agarose gel electrophoresis with ethidium bromide staining.

For reverse transcription, total RNA was first denatured at 94° for 10 min and immediately chilled on ice. First-strand cDNA synthesis was then carried out with total RNA in 50 mM Tris-HCl buffer (pH 8.3) containing 200 units of Moloney murine leukemia virus reverse transcriptase, 26.5 μM oligo(dT), 1 mM dNTP, 20 units of RNasin, 10 mM dithiothreitol, 75 mM KCl, 3 mM $MgCl_2$ in a final volume of 20 μl. The sample was incubated for 10 min at 23° followed by 2 hr at 39°. The reverse transcriptase mixture was then chilled on ice and diluted 2-fold with sterile water in order to avoid *Taq* polymerase inhibition.

PCR reaction was performed according to the method of Dukas *et al.* (21) with 5 μl of reverse transcribed total RNA initially denatured at 99° for 10 min, 1.25 units of *Taq* polymerase, specific sense and antisense primers (1 μM for SSTR1-SSTR3, 0.3 μM for β actin), 250 μM of dNTPs, in 10 mM Tris-HCl buffer (pH 9) containing 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% Triton X-100 in a final volume of 50 μl. The mixture was overlaid with 50 μl of mineral oil to prevent evaporation during incubation and subjected to amplification reaction on a DNA Thermal Cycler (Techné) for varying sequential cycles with denaturation at 94° for 1 min, annealing at 54° (except for SSTR3, annealing at 71°) for 1 min, and extension at 72° for 1.5 min. The amplification was terminated by a final extension step at 72° for 10 min.

The following pairs of primers were used to amplify each cDNA: SSTR1 sense primer, 5′-GCAACATGCTCATGCC-3′, corresponding to nucleotides 623–638 and antisense primer, 5′-GCGTTATCCATCCAGC-3′, corresponding to nucleotides 1022–1037 of the coding sequence of rat SSTR1 (22); SSTR2 sense primer, 5′-GGGCGAATCCGGGGCA-3′, corresponding to nucleotides 961–976 and antisense primer, 5′-GTTTGGAGGTCTCCATTG-3′, corresponding to nucleotides 1450–1467 of coding sequence of rat SSTR2 (23); SSTR3 sense primer, 5′-CAGGCACCCGCTTGCCAGCGG-3′, corresponding to nucleotides 750–770 and antisense primer, 5′-AGGCCGTTGCTGCTGTCCACTGG-3′, corresponding to nucleotides 1181–1203 of coding sequence of rat SSTR3 (24). The resulting cDNA amplification products for SSTR1, SSTR2, and SSTR3 were predicted to be 415, 507, and 454 bp in length, respectively.

PCR amplification of β actin served as an external control. The sense primer, 5′-TCATGCCATCCTGCGTCTGGACCT-3′, corresponding to nucleotides 506–529 and the antisense primer, 5′-CCGGACTCATCGTACTCCTGCTTG-3′, corresponding to nucleotides 998–1021 were defined following cDNA base sequence (25), and the cDNA amplification product was predicted to be 517 bp in length.

PCR amplification of both target samples and β actin were run simultaneously. 5 μl of each PCR sample were analyzed with pGEM DNA markers on 7.5% polyacrylamide gel. Gels were stained with ethydium bromide and exposed to UV light, and quantification was determined by image analysis (Biocom apparatus). In order to standardize the results, the intensity of each PCR product was evaluated by comparison with that of the 517-bp marker used as standard. To correct any variation in RNA content and cDNA synthesis in different preparations, each sample was normalized on the basis of its β actin content. For autoradiographic analysis, 10 μCi of [α-^{33}P]dCTP were added in

each PCR sample, and PCR reaction was performed as described. After polyacrylamide gel electrophoresis, gels were dried and exposed for 48 hr with Kodak X-Omat AR films.

For relative quantification of mRNA levels, PCR reactions were performed with increasing amounts of cDNA template (75–750 ng) to test the relationship between the amount of template and the corresponding PCR product. The exponential phase of amplification was then determined by carrying out the reactions at increasing numbers of cycles for defined amounts of cDNA. According to these results, the following procedure was routinely used. 200 ng of cDNA were amplified by 35 cycles with SSTR1 and SSTR3 sequence-specific primers, and 100 ng were amplified by 25 cycles with SSTR2 and β actin sequence-specific primers.

DNA sequencing. The PCR products for SSTR1 and SSTR2 were separated by agarose gel electrophoresis, excised, and purified with Geneclean II procedure. DNA sequencing was then carried out by the dideoxynucleotide chain-termination procedure (26) using the dsDNA Cycle Sequencing System kit.

Statistical analysis. Statistical comparisons between control and peptide-treated cells were performed using the Student's paired *t*-test.

Results

Effect of EGF and gastrin on [^{125}I]Tyr^{3}SMS binding to AR42J cells. When AR42J cells were incubated in serum-free medium in the presence of EGF or gastrin at 10^{-8} M, in agreement with previous results (5, 17), we observed that the two agents elicited a significant increase in cell growth after 24 hr (EGF: +23 ± 3%; gastrin: +27 ± 3%) and 48 hr (EGF: +64 ± 7%; gastrin: +32 ± 6%) (mean ± S.E., $n = 3$, $p < 0.05$) of treatment. In these conditions, EGF and gastrin increased [^{125}I]Tyr^{3}SMS binding by 31 ± 4% and 47 ± 9%, respectively, after 24 hr of peptide treatment (Fig. 1). The gastrin receptor antagonist (17), CR 1409 at 10 μM, had no effect alone but inhibited by 68 ± 1% the increase of [^{125}I]Tyr^{3}SMS binding induced by gastrin after 24 hr of treatment (Fig. 1). We also tested the effect of basic fibroblast growth factor, a mitogenic peptide for AR42J cells (19). Treatment of AR42J cells for 48 hr in the presence of 0.1 nM basic fibroblast growth factor also induced an increase of [^{125}I]Tyr^{3}SMS binding of 44 ± 12% (mean ± S.E., $n = 3$, $p < 0.05$). The increase in [^{125}I]Tyr^{3}SMS binding did not result from a change in cell density, because similar stimulatory effects of EGF and gastrin were obtained when cells were grown at different densities (from 10,000 to 80,000 cells/cm^2) (data not shown). The EGF- and gastrin-induced increase of [^{125}I]Tyr^{3}SMS binding was not a reflection of increased protein synthesis because treatment of cells for 24 and 48 hr with serum decreased [^{125}I]Tyr^{3}SMS binding by 26 ± 4% and 32 ± 6% ($n = 7$), respectively ($p < 0.05$) (Fig. 1).

The effect of EGF and gastrin was time dependent. At 3, 6, and 12 hr, no significant effect was observed, but a significant increase of [^{125}I]Tyr^{3}SMS binding was observed at 24 hr and maximally occurred at 48 hr after EGF (+44%) or gastrin (+83%) treatment (Fig. 2).

The binding of [^{125}I]Tyr^{3}SMS to somatostatin receptors exhibited a dose-dependent increase in response to a 48-hr treatment with both EGF or gastrin (Fig. 3). The half-maximal effect on [^{125}I]Tyr^{3}SMS binding was obtained at 0.2 ± 0.03 nM EGF and 0.3 ± 0.15 nM gastrin and maximal effect occurred at 10^{-8} M for both peptides.

To clarify the effect of EGF and gastrin on [^{125}I]Tyr^{3}SMS binding, we examined whether somatostatin receptor number or affinity was altered. Scatchard analysis of the binding data revealed that AR42J cells possessed a single class of high affinity binding sites for [^{125}I]Tyr^{3}SMS as previously demonstrated (5, 18). EGF or gastrin treatment of cells for 48 hr resulted in a significant increase in the number of somatostatin receptors without any change in binding affinity (Fig. 4) (Table 1). The increase in the number of somatostatin receptors was

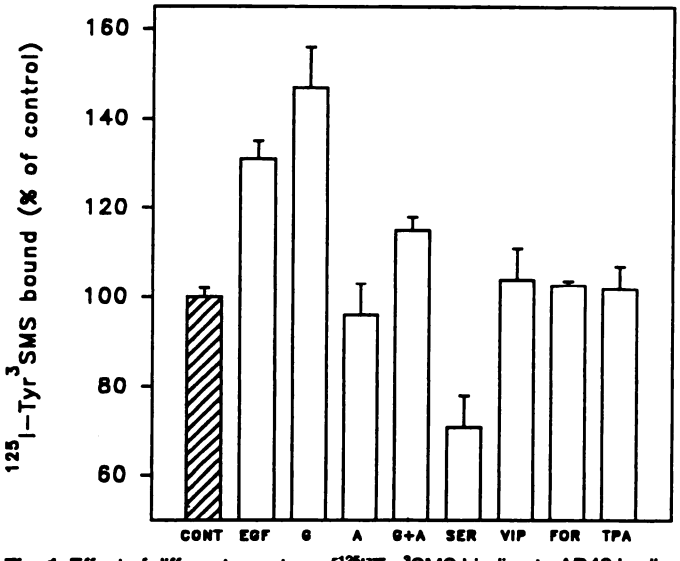

Fig. 1. Effect of different agents on [^{125}I]Tyr^{3}SMS binding to AR42J cells. Cells were cultured in serum-free DMEM and treated with or without (control, *CONT*), 10 nM EGF, 10 nM gastrin (*G*), 10 μM CR 1409 (*A*), 10 nM gastrin + 10 μM CR 1409 (*G + A*), 1 nM VIP, 25 μM forskolin (*FOR*), 50 nM TPA or 10% serum (*SER*) for 24 hr. Then cells were incubated for 75 min at 25° with 100 pM [^{125}I]Tyr^{3}SMS. The amount of specific bound radioactivity was determined as described under Materials and Methods. Values are the mean ± S.E. of triplicate experiments and are expressed as the percentage of control binding obtained from untreated cells.

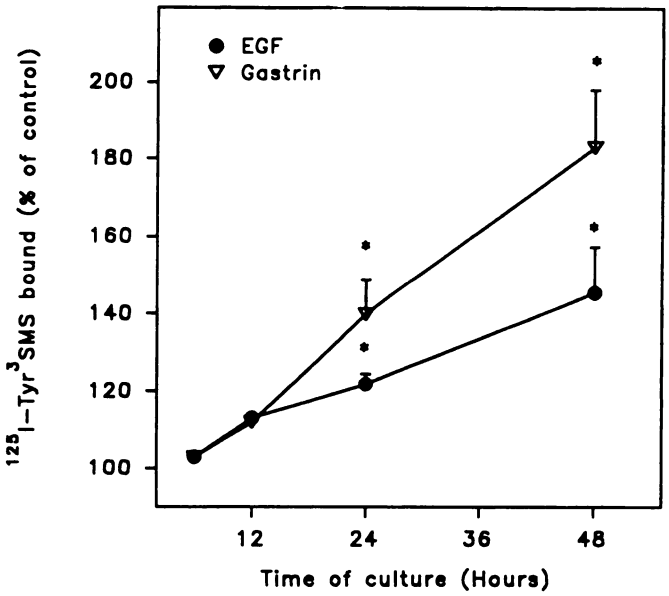

Fig. 2. Time course of the effect of EGF and gastrin on [^{125}I]Tyr^{3}SMS binding to AR42J cells. Cells were treated with or without 10 nM EGF or gastrin. At indicated times, [^{125}I]Tyr^{3}SMS binding was determined as described under Materials and Methods. Values, expressed as the percentage of control value obtained from untreated cells, are mean ± S.E. of five separate experiments performed in triplicate. The statistical significance between control and treated cells is indicated by an *asterisk*, $p < 0.02$.

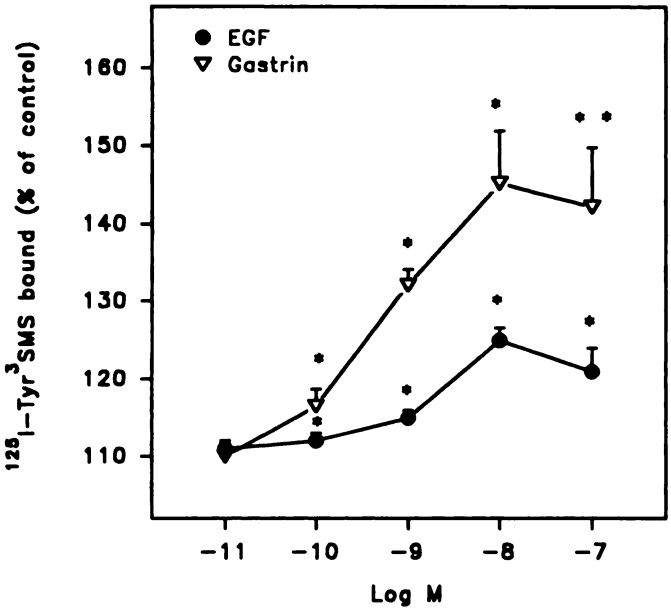

Fig. 3. Dose-dependent effect of EGF and gastrin on [^{125}I]Tyr^{3}SMS binding to AR42J cells. Cells were treated with or without various concentrations of EGF or gastrin for 48 hr and then [^{125}I]Tyr^{3}SMS binding was determined as described under Materials and Methods. Values are the mean ± S.E. of three separate experiments performed in triplicate, and data are expressed as the percentage of control value obtained from untreated cells. The statistical significance between control and treated cells is indicated as follows: * $p < 0.05$, * * $p < 0.02$.

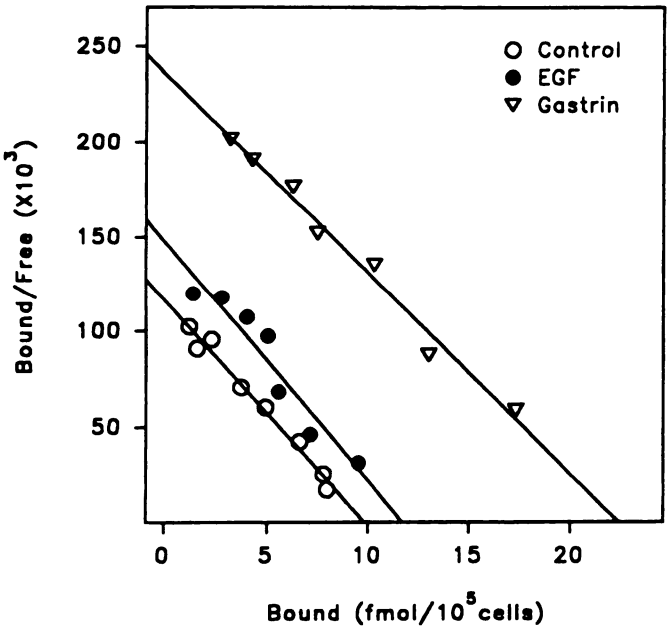

Fig. 4. Scatchard analysis of [^{125}I]Tyr^{3}SMS binding to AR42J cells treated or not with EGF or gastrin. Cells were treated for 48 hr with or without (control) 10 nM EGF or gastrin. Then cells were incubated for 75 min at 25° with 100 pM [^{125}I]Tyr^{3}SMS and increasing concentrations of SMS. Results are presented as a Scatchard plot derived from the binding data of a given experiment, which is representative of at least four separate experiments.

more important after gastrin (+80%) than after EGF (+27%) treatment.

Effect of EGF and gastrin on somatostatin-induced inhibition of cAMP formation. To assess the functional consequences of increase in somatostatin binding, experiments were designed to examine the effect of somatostatin on vasoactive intestinal peptide (VIP)-stimulated cAMP formation in control and EGF- or gastrin-treated cells. As previously observed (5), somatostatin completely inhibited VIP-stimulated cAMP formation, and half-maximal inhibition was obtained with 25 ± 1 pM somatostatin (Fig. 5). When cells were pretreated for 48 hr with 10 nM EGF or gastrin, the stimulatory effect of VIP on cAMP formation was not modified, and somatostatin completely inhibited VIP-stimulated cAMP content. However, EGF or gastrin treatment of AR42J cells shifted to the left the somatostatin dose-response curve. This effect is not large, but it is reproducible, and half-maximal inhibition of VIP-stimulated cAMP formation was observed with concentrations of somatostatin significantly lower in cells pretreated with EGF (IC_{50}, 13 ± 1.5 pM) and gastrin (IC_{50}, 9 ± 2 pM) than in control cells.

Effect of EGF and gastrin on SMS-induced inhibition of cell growth. Previous studies have shown that somatostatin

TABLE 1

Effect of EGF and gastrin on [^{125}I]Tyr^3 SMS binding parameters

AR42J cells were treated or not (control) with 10 nM EGF or gastrin for 48 hr. Then cells were incubated for 75 min at 25° with 100 pM [^{125}I]Tyr^{3}SMS and increasing concentrations of SMS. The K_d and B_{max} binding parameters were determined as described under "Materials and Methods". Results are the mean ± S.E. of five (EGF) or four (gastrin) separate experiments. The statistical significance between control and treated cells is indicated by an asterisk, $p < 0.05$.

Treatment	K_d	B_{max}
	nM	fmol/10^6 cells
Control	0.32 ± 0.03	97 ± 8
EGF	0.34 ± 0.03	121 ± 7*
Control	0.36 ± 0.02	102 ± 10
Gastrin	0.39 ± 0.03	186 ± 14*

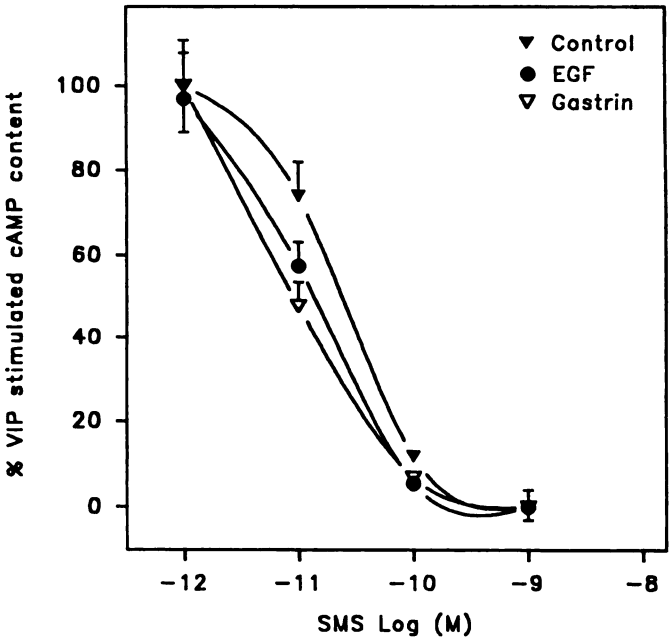

Fig. 5. Inhibition of VIP-stimulated cAMP accumulation by somatostatin in cells pretreated by EGF and gastrin. Cells were treated or not (control) with 10 nM EGF or gastrin for 48 hr. Then cells were incubated for 30 min at 25° with 0.1 μM VIP in the presence or not of increasing concentrations of somatostatin. The cells were washed, and cAMP was extracted and analyzed by radioimmunoassay. Data are expressed as percentage of control. The values are the mean ± S.E. of three different experiments.

inhibits cell proliferation (5). To investigate the functional relationship between the increased somatostatin receptor density and the ability of somatostatin to inhibit cell proliferation, we tested the effect of somatostatin analog, SMS, on growth of AR42J cells cultured in control conditions (without serum) or in the presence of 10 nM EGF or gastrin for 48 hr. When cells were grown in medium lacking serum, growth was increased by about 2-fold in 48 hr. Addition of SMS produced only a small decrease in cell proliferation (Fig. 6), and half-maximal inhibition was achieved with 0.15 ± 0.06 nM SMS. When cells were grown in medium containing 10 nM EGF or gastrin for 48 hr, addition of SMS induced a dose-dependent inhibition of EGF- and gastrin-stimulated cell proliferation. In both cases, 1–10 nM SMS completely suppressed the mitogenic effect of EGF or gastrin and half-maximal inhibition of EGF, and gastrin-stimulated cell growth was produced by 24 ± 6 pM and 25 ± 5 pM SMS, respectively. These results clearly show that EGF or gastrin treatment induces functional receptors for somatostatin and renders the cells more responsive in terms of potency and efficacy to the inhibitory effect of SMS on cell proliferation.

Role of protein kinase C and cAMP pathways on [^{125}I]Tyr^{3}SMS binding. An early event induced by the binding of EGF and gastrin to their respective receptors is the stimulation of inositol phospholipid hydrolysis leading to generation of two second messengers, inositol phosphates and diacylglycerol (27, 28), the latter messenger being known to activate protein kinase C. To investigate the role of protein kinase C pathway in the up-regulation of somatostatin receptors, cells were treated for different times (30–60 min, 24 hr) with 50 nM of the phorbol ester TPA (an activator of TPA-inducible protein kinase C) before binding assay. Whatever the time of treatment, TPA neither modified [^{125}I]Tyr^{3}SMS binding (Fig. 1) nor potentiated the effect of EGF or gastrin on [^{125}I]Tyr^{3}SMS binding (data not shown).

EGF has been demonstrated to modulate adenylate cyclase system in different cell types (29), and gastrin inhibits cAMP accumulation in AR42J cells (30). To test the role of cAMP in the increase of [^{125}I]Tyr^{3}SMS binding induced by EGF and gastrin, control and EGF- or gastrin-stimulated AR42J cells were treated for 24 hr in the presence of forskolin (25 μM), a direct activator of adenylate cyclase. No modification on [^{125}I]Tyr^{3}SMS binding was detected in either control or stimulated cells (Fig. 1). Furthermore, VIP, a nonmitogenic factor for AR42J cells that increased intracellular cAMP levels in this cell line (18), did not modify the [^{125}I]Tyr^{3}SMS binding to AR42J cells after treatment of cells for 24 hr (Fig. 1) or 48 hr (data not shown).

Effect of cycloheximide on EGF- and gastrin-stimulated [^{125}I]Tyr^{3}SMS binding. Because a lag phase was observed between the time of EGF and gastrin exposure and the time of increased [^{125}I]Tyr^{3}SMS binding, we examined the possible requirement of ongoing protein synthesis for somatostatin receptor up-regulation. AR42J cells were treated with the translation inhibitor cycloheximide (1 μg/ml) for 24 hr. In these conditions, the protein synthesis was inhibited by 95% (not shown). Cycloheximide did not modify control [^{125}I]Tyr^{3}SMS binding but completely abolished the stimulatory effect of EGF and gastrin on the binding (Fig. 7). These results suggest that the increase of somatostatin receptors in response to EGF or gastrin required synthesis of new protein(s).

Effect of EGF and gastrin on steady-state level of somatostatin receptor mRNAs. In order to determine whether the up-regulation of somatostatin receptors is due to change in somatostatin receptor mRNA levels, we analyzed the effect of EGF and gastrin on the steady-state level of mRNAs encoding the SSTR1, SSTR2, and SSTR3 somatostatin receptor subtypes by reverse transcription polymerase chain reaction technique. The reverse transcription polymerase chain reaction

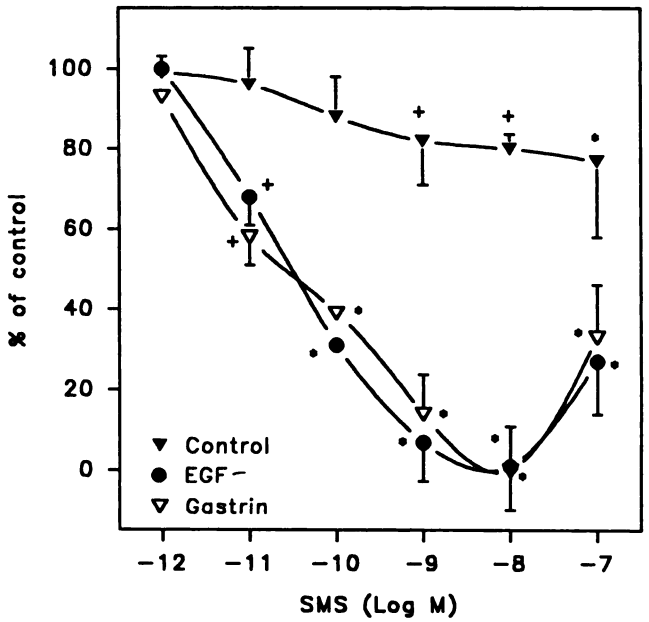

Fig. 6. Concentration-dependent inhibition by SMS of AR42J cell proliferation stimulated by EGF and gastrin. AR42J cells were treated with or without control 10 nM EGF or gastrin for 48 hr in the presence or not of increasing concentrations of SMS. Data are expressed as percentage of the control value or value obtained with EGF- or gastrin-treated cells and are the mean ± S.E. of three separate experiments. The statistical significance of the SMS effect is indicated as follows: + $p < 0.05$, * $p < 0.01$.

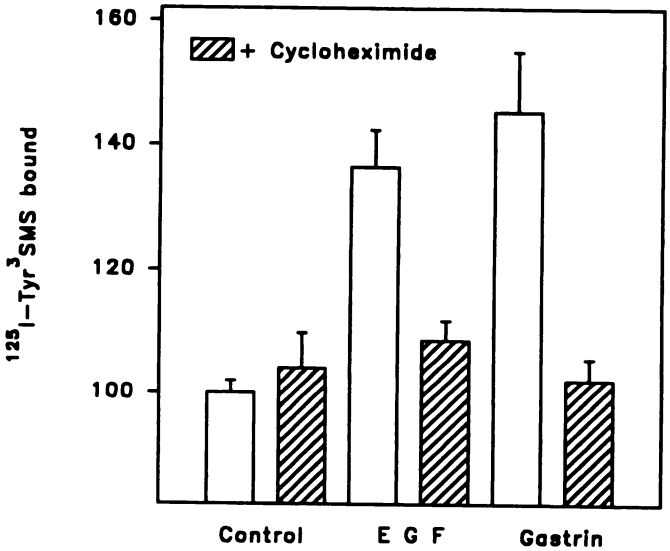

Fig. 7. Inhibition of EGF and gastrin-induced stimulation of [^{125}I]Tyr^{3}SMS binding to AR42J cells by cycloheximide. Cells were treated or not (control) with 10 nM EGF or gastrin in the presence or not of cycloheximide (10 ng/ml) for 24 hr. Then the binding of [^{125}I]Tyr^{3}SMS was determined as described under Materials and Methods. Results are the mean ± S.E. of three separate experiments.

assay yielded specific signals of predicted sizes for SSTR1–SSTR3 mRNAs in control AR42J cells. However, the abundance of PCR products was different according to the somatostatin receptor subtype. A high level of SSTR2 PCR products was detected in AR42J cells, whereas very low levels were obtained for SSTR1 and SSTR3. The identity of the AR42J cell signals was confirmed by sequencing the PCR products for SSTR1 and SSTR2, and we observed identical sequences to that of rat SSTR1 (22) and SSTR2 (23) cDNA. For SSTR3, the PCR product was digested with restriction enzymes that cut once within the fragment (24), and the analysis of digestion products through polyacrylamide gels showed that the PCR product contained the expected restriction sites (data not shown). A typical polyacrylamide gel of PCR products corresponding to SSTR2 is illustrated in Fig. 8, and the analysis by ethidium bromide staining (Fig. 8*A*) and autoradiography (Fig. 8*B*) showed a single band of predicted size at 507 bp. Then, we investigated the effect of EGF and gastrin on SSTR1–3 mRNA levels. Addition of 10 nM EGF or gastrin in the AR42J cell culture medium for 24 hr led to an accumulation of SSTR2 mRNAs (Fig. 8). The time course study of SSTR2 mRNA level in response to EGF and gastrin treatment showed that no significant increase occurred before 24 hr of EGF treatment and 12 hr of gastrin treatment (Fig. 9). EGF had a lower effect than gastrin, because, after a 24-hr peptide treatment, SSTR2 mRNA level was increased by 72 ± 15% ($p < 0.05$) with EGF and by 199 ± 10% ($p < 0.01$) with gastrin. The changes in SSTR2 mRNA levels following EGF or gastrin treatment were observed in the absence of any change in the mRNA level of the β actin, demonstrating the specificity of the effect. Concerning the PCR product for SSTR1 and SSTR3, a faint band was detectable from control cells (Fig. 10). Addition of 10 nM EGF or gastrin in the AR42J cell culture medium for 24 hr did not change the steady-state level of SSTR1 mRNA but increased the level of SSTR3 mRNAs by 327 ± 23% and 290 ± 35% (mean ± S.E., $n = 3$), respectively (Fig. 10).

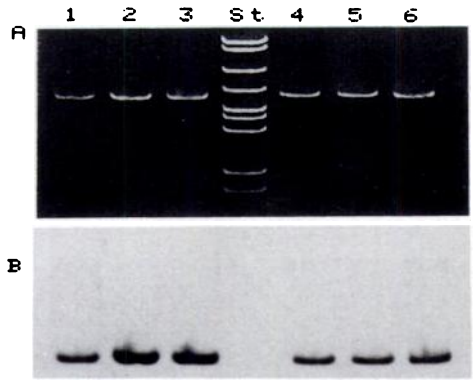

Fig. 8. Analysis of PCR products for SSTR2 mRNA. AR42J cells were treated for 24 hr with or without (*lanes 1* and *4*) 10 nM gastrin (*lanes 2* and *5*) or EGF (*lanes 3* and *6*). Then 100 ng of cDNA were amplified with specific primers for SSTR2 (*lanes 1–3*) or β actin (*lanes 4–6*). The resulting PCR products were analyzed by polyacrylamide gel electrophoresis and ethidium bromide staining (*part A*) or autoradiography of labeled products (*part B*). DNA size markers (*lane St, part A*) indicate 1605, 1198, 676, 517, 460, 396, 350, 222, and 179 bp, from *top* to *bottom*. PCR products appeared as a single band with the predicted size of 507 bp for SSTR2 mRNA (*lanes 1–3*) and 514 bp for β actin mRNA (*lanes 4–6*).

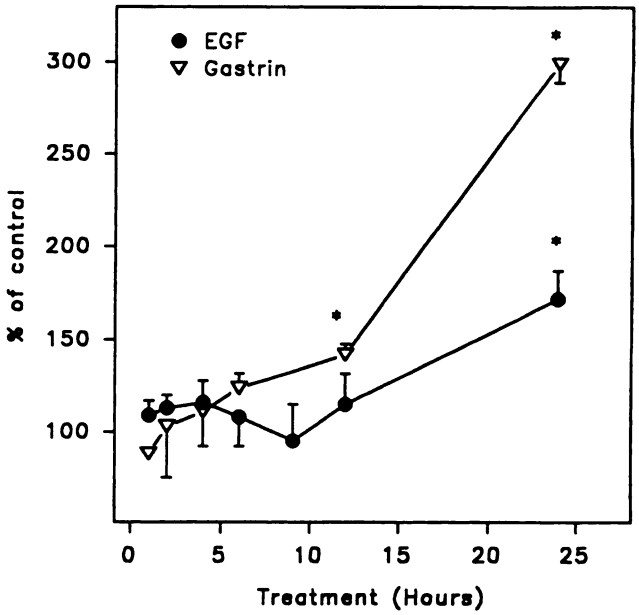

Fig. 9. Effect of EGF and gastrin on the steady-state level of SSTR2 mRNA in AR42J cells. Cells were treated with 10 nM EGF or gastrin. At indicated times, mRNA-derived cDNAs were amplified with specifics primers for SSTR2, and the resulting PCR products were quantified from ethidium bromide-stained polyacrylamide gels. Values are the mean ± S.E. of triplicate experiments and are expressed as the percentage of control value obtained from untreated cells. The statistical significance between control and treated cells is indicated by an *asterisk*, $p < 0.05$.

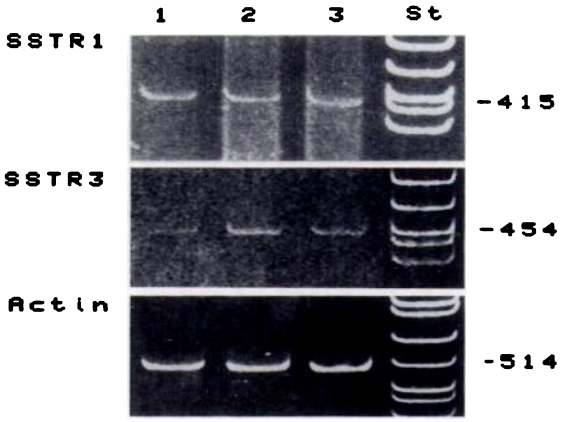

Fig. 10. Effect of EGF and gastrin on the steady-state level of SSTR1 and SSTR3 mRNAs in AR42J cells. Cells were treated with or without (*lane 1*) 10 nM gastrin (*lane 2*) or EGF (*lane 3*) for 24 hr. Then mRNA-derived cDNAs were amplified with specific primers for SSTR1, SSTR3, or β actin, and the resulting PCR products were analyzed by polyacrylamide gel electrophoresis and ethidium bromide staining. PCR products appeared as a single band with the predicted size for SSTR1 (415 bp), SSTR3 (454 bp), and β actin (514 bp). DNA size markers (*lane St*) indicate 1198, 676, 517, 460, 396, and 350 bp from *top* to *bottom*.

Discussion

In the last decade, in addition to the regulatory role of growth factors and hormones on normal and neoplastic cell proliferation, it has become evident that cross-regulation of growth factor or hormone receptors is important in the control of initiation, degree, and duration of growth (14). In this study, we have shown that both EGF and gastrin stimulated the growth of pancreatic tumor AR42J cells as previously reported (5, 17) and that these mitogenic peptides induced a time- and

dose-dependent increase of somatostatin binding to AR42J cells. This was due to a change in receptor number without alteration of affinity and resulted in an increase in functional receptors.

The increase of somatostatin receptor number was observed at EGF and gastrin concentrations involving receptor occupancy and in accordance with the mitogenic effect of the two factors on AR42J cells. Indeed, there is a good relationship between the doses of EGF or gastrin required to stimulate somatostatin receptor binding (ED_{50}, 0.2 and 0.3 nM, respectively) and cell proliferation (ED_{50}, 0.6 and 1 nM, respectively) and to inhibit ligand binding (receptor K_d, 0.9 and 1 nM, respectively) (5, 17). In AR42J cells, the two CCKA and CCKB receptor subtypes have been identified, and the cells possess a large number of CCKB receptors (31). The fact that the CCKB receptor antagonist, CR1409, inhibited gastrin-stimulated somatostatin binding suggests that the CCKB receptor is involved in the up-regulation of somatostatin receptors and is in agreement with the role of this receptor in mediating the proliferative effect of gastrin (17).

EGF and gastrin induced an increase of functional somatostatin receptors. Indeed, AR42J cells treated with EGF or gastrin showed an increase in somatostatin sensitivity. This was first manifested by an increased potency of somatostatin for inhibiting adenylate cyclase, suggesting that, at least in part, EGF- and gastrin-induced somatostatin receptors are coupled to adenylate cyclase. Second, when AR42J cell growth was stimulated with EGF or gastrin, somatostatin analog SMS was more potent and more effective in blocking cell proliferation than in control cells. As previously observed (5), SMS completely antagonized the stimulatory effect of EGF on cell proliferation. Our results also demonstrated that SMS antagonized the effect of gastrin, and this introduces a novel growth factor, the mitogenic activity of which can be completely suppressed by SMS, in tumoral pancreatic cells. We previously reported that in AR42J cells grown in the presence of serum (5), which decreased the somatostatin binding, the antiproliferative effect of SMS was low and similar to that observed in control cells. All these results indicate that SMS is more effective as an antiproliferative agent after induction of cell proliferation by EGF and gastrin, and this could be due to the specific up-regulation of functional somatostatin receptors involved in the antiproliferative effect of the peptide.

EGF and gastrin are known to bind and activate receptors coupled to different signaling pathways. EGF activates receptors that possess intrinsic protein kinase activity (32) and initiates a chain of tyrosine phosphorylation of multiple cellular substrates, including phospholipase C-γ, leading to the generation of inositol phosphate and diacylglycerol, the natural activator of protein kinase C. In certain cells, EGF can also stimulate cAMP formation (29). Gastrin transduces signal through G protein-coupled receptors, which stimulate phospholipase C, causing the breakdown of phosphoinositides and the increase in cytosolic Ca^{2+} and diacylglycerol (28). Gastrin has been also reported to inhibit adenylate cyclase (30) and to stimulate a tyrosine kinase activity (33). Concerning the signal transduction pathways involved in somatostatin receptor up-regulation, the present studies demonstrate that neither agents that raise intracellular cAMP nor TPA induces an increase of somatostatin binding, suggesting that in AR42J cells, protein kinase A and TPA-sensitive protein kinase C signaling pathways are not involved in somatostatin receptor up-regulation. Further studies are needed to identify specific pathways that are used by EGF and gastrin to up-regulate somatostatin receptors.

The timing of growth factor-induced somatostatin receptor up-regulation suggests that EGF and gastrin act at the genomic level. The protein synthesis inhibitor, cycloheximide, totally suppressed the effect of EGF and gastrin on somatostatin binding and may reflect the inhibition of the translation of somatostatin receptor or of a protein that regulates the somatostatin receptor expression. The recent cloning of genes for different somatostatin receptor isoforms (12) suggests that distinct somatostatin receptors could be expressed in AR42J cells and independently regulated by heterologous factors. Our data showed that at least three somatostatin receptor subtype mRNAs, SSTR1, SSTR2, and SSTR3, are present in AR42J cells. As previously reported (23), SSTR2 mRNA was detected at a high level. This is in agreement with the findings that AR42J somatostatin receptors displayed the same ligand binding properties as did the cloned SSTR2 receptor subtype that is a high affinity for somatostatin and somatostatin analog SMS (5, 23). We found that the expression level of SSTR1 mRNA was not modulated by treatment of AR42J cells with EGF or gastrin. By contrast, both EGF and gastrin induced an increase in SSTR2 mRNA levels. There is a good relationship between the relative efficacy of EGF and gastrin to enhance the expression levels of SSTR2 mRNA and to increase the number of somatostatin receptors, gastrin being the most active. SSTR3 mRNA levels were also increased after EGF and gastrin treatment. However, the absence of specific SSTR3 receptor agonist precludes the analysis of the effect of EGF and gastrin at the SSTR3 receptor binding level. Further studies are required to determine whether the increased mRNA levels reflect an increase of transcriptional efficiency of SSTR2 and SSTR3 genes and/or mRNA stability. By contrast to the early responses induced by growth factors, such as the stimulation of the expression of "immediate-early" genes shown in a number of cell lines (34), stimulation of SSTR2 mRNA expression may be regarded as a delayed response, because the effect started several hours after EGF or gastrin treatment.

The physiological meaning of EGF- and gastrin-induced functional somatostatin receptor up-regulation is not known. The increased receptor expression could be secondary to the mitogenic signal of the peptides and could represent a part of a homeostatic cellular program in response to proliferative stimuli. Indeed, EGF and gastrin are known to be trophic factors for gastrointestinal tract and pancreas, whereas somatostatin is a negative regulator (35). The presence of these peptides in the normal pancreas (36, 37) and the high expression of gastrin and somatostatin in the fetal pancreas (37, 38) support the idea of a role of these factors in the early stages of pancreatic development and suggest that the control of somatostatin receptor level by trophic factors may have a critical role in the construction of normal tissue during organogenesis. The disruption of such a control mechanism may result in increased growth potential and may have an important role in the pathogenesis of certain tumors.

In summary, we found that two mitogenic factors, EGF and gastrin, up-regulate functional somatostatin receptors. This effect leads to an increased responsiveness of cells to the inhibitory effect of somatostatin on adenylate cyclase and cell

proliferation and results, at least in part, in the increase of the mRNA levels of SSTR2 and SSTR3 somatostatin receptor subtypes. This response is a delayed event that can represent a cellular mechanism regulating EGF and gastrin mitogenic activities.

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