Stimulation by somatostatin of dephosphorylation of membrane proteins in pancreatic cancer MIA PaCa-2 cell line

Marion T. Hierowski*+, Charles Liebow+°, Karen du Sapin+ and Andrew V. Schally*

*Endocrine and Polypeptide Laboratories, Veterans Administration Medical Center, Department of Medicine, Tulane University School of Medicine, New Orleans, LA 70146, +Department of Physiology, Louisiana State University Medical Center, New Orleans, LA 70119, and °Department of Oral Surgery, State University of New York at Buffalo, School of Dentistry, Buffalo, NY 14214, USA

Received 6 November 1984

A membrane receptor and a cytosolic receptor for somatostatin were found in a human undifferentiated pancreatic cancer cell line (MIA PaCa-2). Binding of somatostatin to this membrane receptor activates dephosphorylation of a phosphotyrosyl-membrane protein whose phosphorylation was promoted by epidermal growth factor (EGF). Vanadate, a purported inhibitor of dephosphorylation, interferes with the action of somatostatin. These findings suggest a possible biochemical mechanism by which somatostatin may inhibit the growth of human pancreatic cancers.

Somatostatin *Somatostatin receptor* *EGF* *EGF receptor* *Dephosphorylation* *Pancreatic cancer* *Growth control*

1. INTRODUCTION

The existence of low-M_r peptide hormones which stimulate cell growth and proliferation is well documented [1,2]. Conversely, little is known about hormones or growth inhibiting factors which have antiproliferative effects on the cell. Recent studies indicate that one of these antiproliferative factors could be somatostatin, a ubiquitous neuropeptide which inhibits numerous cellular processes [3–5]. For instance, it was recently demonstrated that somatostatin inhibited EGF-induced centrosomal separation, DNA synthesis, and cell replication in HeLa cells [6]. Inhibition of growth of pancreatic carcinoma in animal models by analogs of somatostatin was also recently demonstrated [7].

The report that somatostatin-14 stimulates cytoplasmic PPPase (phosphoprotein phosphatase) in normal pancreas [8] prompted us to study the effect of somatostatin on dephosphorylation of membrane proteins in the MIA PaCa-2 cells from human pancreatic adenocarcinoma. Here, we specifically concentrated on the relation of somatostatin and its receptor to EGF receptor phosphorylation-dephosphorylation process. The EGF receptor is a 170-kDa membrane glycoprotein that contains intrinsic tyrosine kinase activity.

Abbreviations: SS, somatostatin; EGF, epidermal growth factor

2. MATERIALS AND METHODS

2.1. *Cell culture*

The MIA PaCa-2 cell line [9], a human pancreatic carcinoma cell line, was obtained from American Tissue Type Cultures. The cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 2.5% horse serum. Cells were incubated at 37°C in humidified air containing 5% CO_2. Cells were harvested using 0.1% trypsin in phosphate-buffered saline. Membrane vesicles from MIA PaCa-2 cells were prepared as in [10].

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

2.2. *Somatostatin binding to its receptor*

[^{125}I]Iodo-Tyr^{1}-somatostatin (Peninsula) was prepared by chloramine-T iodination. One microgram of Tyr^{1}-somatostatin in 25 μl of 0.01 N acetic acid was added to 50 μl of 0.5 M phosphate buffer, pH 7.5, containing 2.0 mCi carrier-free Na^{125}I. After addition of 1 μg chloramine-T, the mixture was agitated for 90 s and the reaction was terminated by the addition of 100 μl saturated tyrosine solution. The labelled somatostatin was purified by chromatography on Biogel P-2 column and eluted with 0.01 N acetic acid containing 0.1% BSA. The specific activity of the labelled material determined by radioligand receptor assay ranged from 1100 to 1900 μCi/g. The [^{125}I]iodo-Tyr^{1} - somatostatin tracer was stored in aliquots at -10°C and was stable for at least 1 month.

2.3. *Somatostatin binding assay*

Aliquots of the membrane suspension containing 50–100 μg protein were incubated in 500 μl of 50 mM Tris–HCl buffer, pH 7.4, containing 5 mM $MgCl_2$, 2 mM EGTA, 20 μg/ml ethylmercurithiosalicylate (Thimerosal) (Sigma, St. Louis, MO) and 50 μg/ml bacitracin for 90 min at 16°C.

After incubation, the receptor-bound and free radioactivity were separated by filtration using Whatman 6F/C glass fiber filters (Whatman, Clifton, NJ). Filters were washed 3 times with 4 ml cold phosphate-buffered saline (PBS) and the bound radioactivity was measured in a Beckman Gamma-spectrometer. The degradation of cold and labelled somatostatin during the binding assay was measured as described [11]. Specific binding was estimated as a difference between 'total' binding (i.e., in the presence of a tracer alone) and 'non-specific' binding which was measured in the presence of 10^{-7} M unlabelled somatostatin. Specific binding represented 1–3% of the total radioactivity. Non-specific binding accounted for 15–20% of total binding.

2.4. *Miscellaneous*

Phosphoprotein phosphatase activity was measured by the release of ^{32}P from ^{32}P-labelled substrates at 37°C. Reaction mixture for the PPPase activities was determined as detailed using ^{32}P-labelled calf thymus type II-S histone (^{32}P-histone) as exogenous substrate [12]. Purification of phosphoprotein-phosphatase from rat pancreas was carried out as described [13].

3. RESULTS

3.1. *Binding of ^{125}I-Tyr^{1}-somatostatin-14 to the membranes and cytosolic fractions from MIA PaCa-2 cells*

The specific binding of tracer was dependent upon incubation time and protein concentration. Maximal equilibrium level of tracer binding was found at 15 min for both cytosolic and membrane fraction. The optimal protein concentration was found to be 150 μg and further increases in the protein concentration had little effect on the nonspecific binding. The addition of increased concentration of unlabelled somatostatin-14 to the incubation mixture resulted in a progressive inhibition of tracer binding (fig.1), indicating the presence of a finite number of sites of competition between labelled and unlabelled tracer. The amount of tracer bound per mg membrane proteins was 3.6 ± 0.4 fmol. The cytosolic sites were substantially more dense than the membrane sites, with

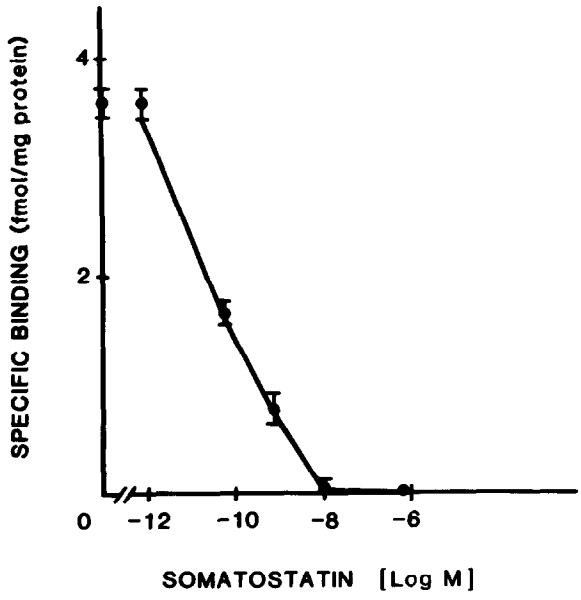

Fig.1. Specific binding of ^{125}I-Tyr^{1}-somatostatin-14 to membranes from MIA PaCa-2 cells. Isolated membranes were incubated with radioactive tracer in the presence of various concentrations of unlabelled somatostatin-14. Mean values ± SE of 3 experiments.

38.0 ± 0.6 fmol tracer bound per mg cytosolic protein (not shown). Since cytosolic fraction represents about 35% of cellular proteins and membrane fraction only 4–5% [13], there appear to be almost two orders of magnitude more cytosolic than membranous binding sites. Consequently, the role of cytosolic phosphoprotein-phosphatases in dephosphorylation of membrane proteins cannot be excluded.

3.2. *Stimulation by somatostatin of phosphoprotein phosphatases in membranes from MIA PaCa-2 cells*

To study the influence of somatostatin on phosphoprotein phosphatases in plasma membranes, we used as a substrate synthetic phospho-Tyr-histones and phospho-Ser-histones prepared as in [14]. Phosphatase activity is present in MIA PaCa-2 cell membrane, for both phospho-Tyr-histones and phospho-Ser-histones, but only phospho-Tyr-histones dephosphorylation is enhanced by somatostatin (fig.2). These data suggest that the membranes contain phosphotyrosine specific phosphatase different from phosphoserine specific protein phosphatase and that only the former is stimulated by somatostatin (fig.2). Phosphoprotein-phosphatase, isolated and partially purified from rat pancreas, is also specific as phosphoprotein-phosphatase from MIA PaCa-2 cell membrane for these two phosphorylated substrates.

Somatostatin caused dephosphorylation of membrane proteins which were previously enzymatically phosphorylated in the presence of EGF in a manner analogous to the dephosphorylation of the synthetic phosphorylated tyrosine and serine substrates (fig.3). The stimulatory effect of somatostatin on dephosphorylation of endogenous MIA

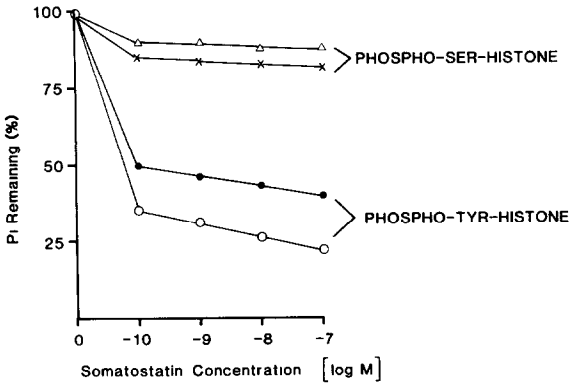

Fig.2. Effect of somatostatin on dephosphorylation of phospho-Tyr-histones and phospho-Ser-histones by membranes from MIA PaCa-2 cells (△, ●) and purified rat pancreatic cytosolic PPPase (×, ○). Protein phosphatase activity was measured in the presence of various concentrations of somatostatin using phospho-Tyr-histone (0.20 μM ^{32}P) (○, ●) or phospho-Ser-histone (0.48 μM ^{32}P) (△, ×).

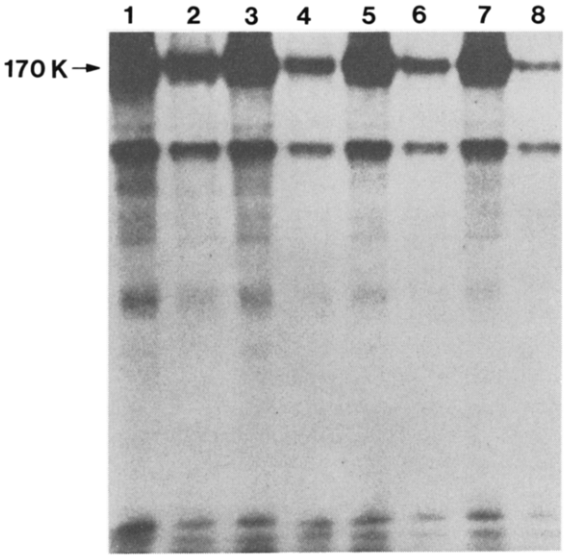

Fig.3. An autoradiograph of an SDS-PAGE showing the dephosphorylation of MIA PaCa-2 membrane proteins in the presence of 10^{-8} M somatostatin (lanes 1, 3, 5, 7) and without somatostatin (controls) (lanes 2, 4, 6, 8). The results are shown as a function of incubation time: Lanes 1 and 2, 10 min; lanes 3 and 4, 20 min; lanes 5 and 6, 30 min; lanes 7 and 8, 60 min. Aliquots of MIA PaCa-2 membranes were phosphorylated by incubation at 0°C in 20 mM Hepes buffer, pH 7.5, containing 20 μM [γ-^{32}P]ATP, 2 mM $MnCl_2$ and 0.5 μg/ml of EGF in a total volume of 100 μl. After 15 min, an aliquot was removed and unlabelled ATP (800 μM) and 10^{-8} M somatostatin were added. In control experiments, somatostatin was omitted. The incubation was continued at 0°C and aliquots were removed at 10, 20, 30 and 60 min and added to a buffer solution containing 0.2 M DTT 6% SDS and 25% glycerol in 50 mM Tris–Cl, pH 7.5. Electrophoresis was carried out in 10% polyacrylamide gel as in [17]. Autoradiography of the stained dried gel was carried out overnight at −70°C in Dupont Cronex cassettes with intensifying screens using Dupont X-ray film.

PaCa-2 membrane proteins was measured as follows: the membranes were phosphorylated with [γ-^{32}P]ATP in the presence of EGF and then the rates of dephosphorylation were measured (in the presence of an excess of non-radioactive ATP to prevent the reincorporation of labelled phosphate) in the presence or absence of 10^{-8} M somatostatin-14. Fig.3 shows that the dephosphorylation of phosphoprotein band of 170 kDa (which represents the EGF receptor with the protein kinase domain) was markedly stimulated in the presence of somatostatin-14 and that the dephosphorylation increased with the time of incubation.

3.3. *The influence of vanadate on the stimulatory effect of somatostatin on dephosphorylation of membrane proteins from MIA PaCa-2 cells*

To determine the effect of vanadate on somatostatin-stimulated dephosphorylation of membrane proteins from MIA PaCa-2 cells, the membranes were phosphorylated with [γ-^{32}P]ATP in the presence or absence of EGF and 10^{-8} M somatostatin as indicated (fig.4). Vanadate (10 μM) was added to the incubation mixture in combination with the EGF and somatostatin. The incorporation of ^{32}P from ^{32}P-labelled ATP was measured as described in fig.2. The mixture, consisting of 20 mM Hepes buffer, pH 7.5, 15 M [γ-^{32}P]ATP, 1 mM $MnCl_2$ and aliquots of membranes from MIA PaCa-2 cells in a final volume of 80 μl, was incubated for various times.

Vanadate could increase the level of phosphotyrosine in the cellular phosphoproteins by inhibiting phosphotyrosyl-protein phosphatase. In contrast, somatostatin stimulated phosphotyrosine phosphatase and decreased the level of phosphotyrosine in the cellular phosphoprotein. The resulting inactivation of tyrosine kinase nullified the proliferation and growth stimuated by EGF. The phosphorylated membrane proteins were incubated with 10^{-8} M somatostatin and increasing amounts of purified cytosolic rat PPPase in the presence or absence of 10 μM vanadate (fig.5). The percentage of P_i released from phosphorylated membrane proteins was dependent on the amount of purified cytosolic rat PPPase added. The membrane-bound PPPase was inhibited by 10 μM of vanadate.

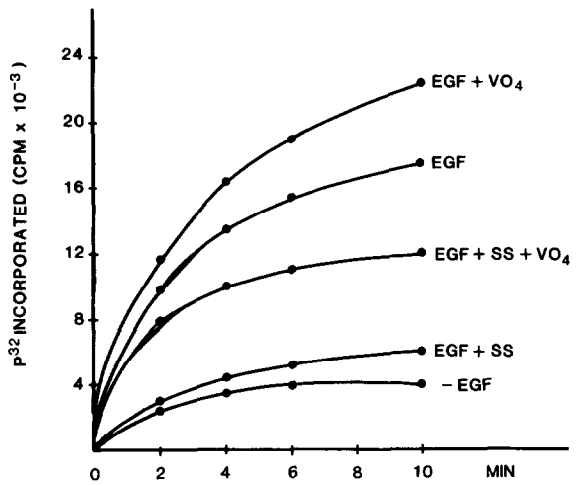

Fig.4. The effects of EGF, somatostatin and vanadate in combination on the protein kinase activity in MIA PaCa-2 cell membranes. Incubation mixtures and conditions are described in the text.

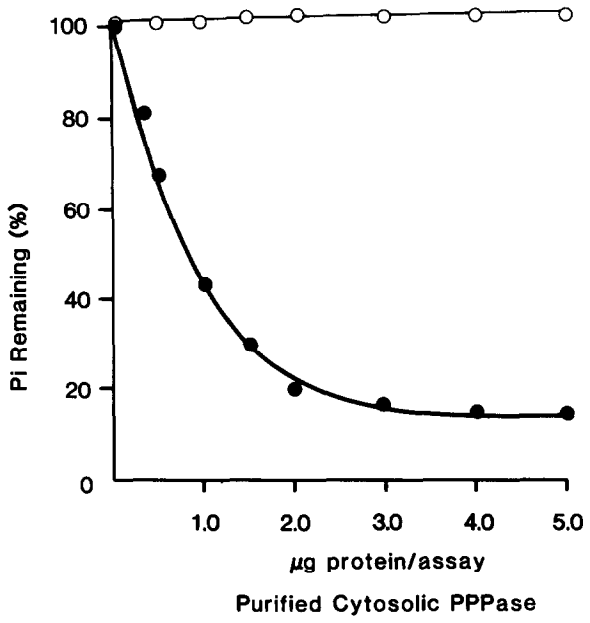

Fig.5. The effect of vanadate (10 μM) on dephosphorylation of membrane proteins in the presence of 10^{-8} M somatostatin. Varying amounts of purified rat cytosolic PPPase were incubated with phosphorylated membrane protein and somatostatin in the presence (○) or absence (●) of 10 μM vanadate. The percentage of labelled phosphate retained by the protein is plotted.

4. DISCUSSION

This study examines the relationship of EGF, somatostatin and vanadate on the process of net phosphorylation of the EGF receptor from human pancreatic cancer. The EGF receptor has 3 functional domains: an EGF binding site located on the external cell surface, and transmembrane and cytoplasmic tyrosine kinase domains. Adenosine triphosphate is a donor for phosphorylation of intracellular substrates by the kinase. Phosphorylation of EGF receptor at tyrosine residues plays an important role in regulation of cellular proliferation and growth of normal tissue. Other human growth factors also regulate cell proliferation through the process of phosphorylation. Somatostatin nullifies the growth stimulation produced by EGF and potentiated by vanadate. Vanadate inhibits a phosphotyrosine specific protein phosphatase, which results in a synergistic effect of vanadate and EGF on DNA synthesis in human fibroblast [15].

Our work shows that somatostatin reverses the stimulatory effect of EGF on phosphorylation of the tyrosine-kinase domain of the EGF-receptor. This suggests that the antagonistic effect of somatostatin on EGF is mediated directly through the phosphorylated product. Evidence exists that somatostatin inhibits cell growth in tissue cultures [6]. Preliminary evidence has suggested that somatostatin analogues also may be capable of inhibiting the growth of exocrine pancreatic cancers in animal models [7]. Our results might explain how such inhibition could be mediated. The antagonistic effect of EGF and somatostatin on the EGF receptor phosphorylation and the presence of EGF and somatostatin receptors on the membranes of a human pancreatic adenocarcinoma suggest a potential biochemical mechanism for mediating this control of growth.

The site of action of somatostatin on the membrane in addition to postmembrane and cytosolic located mechanisms indicate the remarkable plurality of binding sites of this hormone in the MIA PaCa-2 cell line (unpublished). The multiplicity of somatostatin binding sites and the widespread distribution of similar sites in the body, together with antiproliferative effects of somatostatin (e.g., on HeLa cells [6]), suggest that somatostatin may belong to a class of hormones that act as endogenous growth inhibitors [16].

Preliminary studies in tissue culture of MIA PaCa-2 cells demonstrate that EGF stimulates human pancreatic adenocarcinoma cell growth. The antiproliferative properties of somatostatin are under investigation (unpublished). The relationship between EGF and somatostatin found in this work in human pancreatic adenocarcinoma cell line suggests a potential clinical application for somatostatin. If this interaction truly regulates cellular growth in situ, the receptor found in this pancreatic adenocarcinoma may have counterparts in other tumors. Attempts could then be made to inhibit pancreatic cancer growth by modern analogs of somatostatin.

ACKNOWLEDGEMENT

This work was supported in part by a grant from the Schlieder Foundation.

REFERENCES

[1] Bradshaw, R.A. and Rubin, J.S. (1980) J. Supramol. Struct. 14, 183–199.
[2] Hochhauser, S.J., Stein, J.L. and Stein, G.S. (1981) Int. Rev. Cytol. 71, 95–243.
[3] McCann, S.M. (1982) Annu. Rev. Pharmacol. Toxicol. 22, 491–515.
[4] Reichlin, S. (1983) New Engl. J. Med. 309, 1495–1501, 1556–1563.
[5] Schally, A.V., Coy, D.H. and Meyers, C.A. (1978) Annu. Rev. Biochem. 47, 89–128.
[6] Mascardo, R.N. and Sherline, P. (1982) Endocrinology 111, 1394–1396.
[7] Redding, T.W. and Schally, A.V. (1984) Proc. Natl. Acad. Sci. USA 81, 248–252.
[8] Reyl-Desmars, F. and Lewin, M.J.M. (1982) Biochem. Biophys. Res. Commun. 109, 1324–1331.
[9] Yunis, A.A., Arimura, G.K. and Russin, D.J. (1977) Int. J. Cancer 19, 128–135.
[10] Cohen, S., Ushiro, H., Stoscheck, C. and Chinkers, M. (1982) J. Biol. Chem. 257, 1523–1531.
[11] Auilera, G., Parker, D.S. and Catt, K.J. (1982) Endocrinology 111, 1376–1384.
[12] Khandelwal, R.L., Vandenheede, J.R. and Krebs, E.G. (1976) J. Biol. Chem. 251, 4850–4858.
[13] Reyl, F.J. and Lewin, M.J.M. (1982) Proc. Natl. Acad. Sci. USA 79, 978–982.
[14] Swarup, G., Cohen, S. and Garbers, D.L. (1981) J. Biol. Chem. 256, 8197–8201.
[15] Carpenter, G. (1981) Biochem. Biophys. Res. Commun. 102, 1115–1121.
[16] Iversen, O.H. (1981) in: Tissue Growth Factors (Baserga, R. ed.) p.491, Springer, New York.
[17] Laemmli, U.K. (1970) Nature 277, 680–685.