JOURNAL OF SURGICAL RESEARCH 57, 619-624 (1994)

Molecular and Functional Studies of Inhibitory G Protein in RINm5F Cells[1]

MICHAEL K. MCLEOD, M.D., PIYUSH C. KOTHARY, M.S., AND CATHLEEN M. FARRELL, B.S.

Department of Surgery, Section of General Surgery-Division of Endocrine Surgery, University of Michigan Medical Center, Ann Arbor, Michigan 48109

Submitted for publication December 1, 1993

Inhibitory G proteins (G_i) play an important role in cell proliferation. In order to characterize G_i proteins in RINm5F (RIN) cells, we first established RIN cells in cell culture. Immunoblot analysis was performed on extracted G proteins using Western blot techniques and a G_i-specific antibody. We identified three prominent bands consistent with three distinct inhibitory α subunits of membrane-bound G protein (G_i) in RIN cells. In contrast, we identified only one prominent distinct inhibitory α subunit of G protein in an equal quantity of membrane-protein in our control (normal rat pancreas). In several cell types, G_i is known to mediate the inhibitory action of somatostatin on intracellular cyclic AMP (cAMP) accumulation. Therefore, we studied the action of the long-acting analogue of somatostatin, octreotide (SMS), on basal and 3-isobutyl-1-methylxanthine-stimulated cAMP accumulation in RIN cells. SMS did not inhibit cAMP accumulation or tritiated thymidine incorporation into DNA (TTID) in RIN cells. However, when treatment with SMS is supplemented with the nonhydrolyzable analogue of guanine nucleotide, Gpp(NH)p (Gpp), which is known to dissociate G proteins into its constitutive subunits, then SMS + Gpp induced an inhibitory action and significantly reduced cAMP accumulation and TTID. These data are consistent with the concept of qualitatively and functionally altered inhibitory G protein expression in the insulin-producing, islet cell (RINm5F) rat insulinoma tumor cell line. Further study of human tumors will lead to new insights into the clinical implications of G protein-mediated signal transduction in insulinoma.

INTRODUCTION

Guanine nucleotide-binding proteins (G proteins) functionally mediate transmembrane signaling pathways that are composed of receptors, G proteins, and effectors. G proteins are heterotrimeric proteins with subunits designated α, β, and γ in the order of their decreasing mass size. As stated, these proteins transduce cell membrane-bound receptor–ligand interactions by serving as signal proteins for chemically and physically coded information [1–4]. G proteins have been shown to link cell surface receptors for biologically active hormones to adenylyl cyclase. The use of cholera toxin and pertussis toxin (PT) have led to the discovery of two distinct G proteins, G_s and G_i. G_s and G_i have been shown to mediate the stimulation and inhibition of adenylyl cyclase activity, respectively [1].

Furthermore, there are three (at least) distinct molecular species of G_i proteins that inhibit adenylyl cyclase activity [5, 6]. These proteins are referred to as $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$ [7, 8]. These specific inhibitory G proteins range in molecular weight between 38 and 45 kDa. G_i plays an important role in cell proliferation and the growth of certain cells and cones in the eye [9–11]. Furthermore, the reaction of the brain–gut peptide, somatostatin (SMS), appears to be mediated primarily by G_i [12, 13]. However, we have shown that SMS does not inhibit RINm5F cell (a rat insulinoma cell line (RIN)) proliferation, and that PT partially facilitated the action of SMS [14]. In the present report, we further describe inhibitory G protein in RIN cells and show that the expression of G_i is altered when compared with normal rat pancreas. We further examined the effect of supplementing growth media for RIN cells with the nonhydrolyzble analogue of guanine nucleotide, Gpp(NH)p, on the ability of SMS to inhibit basal and 3-isobutyl-1-methylxanthine (IBMX)-stimulated intracellular cyclic AMP (cAMP) accumulation, as well as on its ability to affect cell proliferation as measured by the rate of tritiated thymidine incorporation into extracted DNA (TTID).

MATERIALS AND METHODS

Chemicals

The longer acting, synthetic somatostatin analogue, octreotide (Sandostatin), was purchased from Sandoz

[1] Presented at the Annual Meeting of the Association for Academic Surgery, Hershey, PA, November 10–13, 1993.

0022-4804/94 $5.00

pharmaceuticals. Roswell Park Memorial Institute 1640 (RPMI 1640) and other chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO). Immunoblot kits were obtained from Bio-Rad (Richmond, CA). [^{3}H]Thymidine was purchased from Amersham (Arlington Heights, IL). Antibodies to G_i (AS/7) were from rabbit antisera and purchased from Du Pont–NEN (Boston, MA).

Establishment of RIN Cell in Culture

As described previously, RINm5F cells were maintained in 75 cm^2 tissue culture flasks at 37°C in 5% CO_2 in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum, 2 m*M* L-glutamine, and 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 trypsin, and subsequently were plated onto five wells in 6-well tissue culture plates and grown in 2 ml of medium supplemented with 1% fetal bovine serum × 48 hr. This was followed by 2 ml of standard medium (RPMI 1640 with 2 m*M* L-glutamine and 1% penicillin and 1% streptomycin) supplemented with 0.5% fetal bovine serum (FBS) × 24 hr. The medium was aspirated and 2 ml of fresh standard 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 [14].

Immunoblot of G Proteins

To characterize G_i in RIN cells and normal rat pancreas, we first excised rat pancreas and minced its tissue in RPMI 1640. Then RIN cells and minced pancreas were homogenized separately with 20–25 strokes in a Dounce tissue homogenizer containing Tris buffer. Tris buffer contained 1 μmole/liter phenylmethylsulfonyl fluoride, 0.1 mg/ml bacitracin, 2 μmole/liter leupeptin (in Tris buffer). The G proteins were extracted from the pellets by centrifuging (2000*g*) at 4°C for 10 min in Tris buffer containing 1% detergent, zwittergent 3-12. The supernatant was further centrifuged at 8000*g*. Protein was estimated using a kit from Bio-Rad. One hundred micrograms of protein was subjected to 12% polyacrylamide gel electrophoresis. The proteins were electrophoretically transferred to nitrocellulose membranes [13]. For immunoblot analysis, the blots were blocked overnight in 3% bovine serum albumin and then incubated in the primary antibody AS/7. The bands were then detected with an alkaline phosphatase reagent from Bio-Rad. Antibody AS/7 is specific for two of the α subunits of G_i, $G_{i\alpha 1}$ and $G_{i\alpha 2}$ [15, 16].

Estimation of DNA Synthesis

DNA synthesis was estimated by the rate of [^{3}H]-thymidine incorporation into extracted DNA as described previously [14]. A modified diphenylamine reaction for desoxypentose was performed to measure DNA content. [^{3}H]Thymidine incorporation into extracted DNA was expressed as cpm/μg DNA.

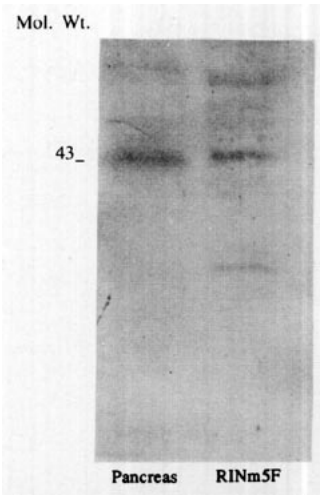

FIG. 1. Immunoblot analysis of G_i α subunits in rat pancreas and RINm5F (RIN) cells. Minced pancreas and RIN cells were treated with 1% zwittergen 3-12 (to extract G proteins; for details, see Materials and Methods). 100 μg of proteins was applied to SDS–PAGE using 12% polyacrylamide gels. The proteins were transferred to nitrocellulose membranes electrophoretically and then immunoblot analysis was performed using antibody AS/7 (specific for G_i α subunit, subtypes of G_i) with a Bio-Rad immunoblot kit. Shown on the ordinate (*y*-axis) is the molecular weight in kilodaltons (kDa). The figure is representative of three experiments. (The blot shown is enlarged 1:5.)

Cyclic Adenosine Monophosphate

RIN cells (10^5 cells) were equilibrated in RPMI 1640 for 15 min. The cells were then treated with reagents for 5 min, vortexed gently, and centrifuged at 2000 *g* for 10 min at 4°C. The pellets were deproteinized with 15% TCA, ether extracted, and lyophilized. Cyclic AMP levels were measured using a specific radioimmunoassay that has been previously described [14].

Gpp(NH)p Supplementation

Studies using Gpp(NH)p (Gpp) to supplement the action of octreotide were performed using untreated RIN cells. The cells were not permeabilized in these experiments. Permeabilization may result in nonphysiological and artificial effects, although the pattern of responses appear to remain consistent in studies in which the cells were permeabilized using TRANSPORT [Gibco (data not included)]. Gpp (10^{-6} *M*) was used in all experiments described in which Gpp was used.

Statistics

All values shown represent the mean ± SEM. Significant differences between treatment groups were tested

TABLE 1

Cyclic AMP Accumulation in Rat Pancreas and RINm5F Cells

Cell type	cAMP (fmole/10^5 cells)
Pancreatic cells	751.8 ± 27
RIN cells	5472 ± 590*

Note. cAMP was measured as described under Materials and Methods. Mixed isolates (acinar and islet cells) were obtained by collagenase digestion at 37°C. Data shown are the means ± SEM for five determinations.

* $P < 0.05$.

for using either the Student's t test or the Wilcoxon signed rank test. A $P \leq 0.05$ was the criterion used to identify a significant difference.

RESULTS

Characterization of G Protein in RINm5F Cells

As shown in Fig. 1, in control (normal rat) pancreas, one band was identified by antibody specific for the G_i α subunits of G protein. In RIN cells, three prominent bands were identified using the same antibody. Both control and RIN cells were observed to contain the same dominant band with an approximate molecular weight (MW) of 43 kDa. This is consistent with G_i. The two additional bands observed in the RIN cells have approximate MW ranging from 39 to 45 kDa.

Comparison of Cyclic AMP Accumulation: Normal Pancreatic vs RINm5F Cells

Cyclic AMP accumulation in pancreatic cells was significantly lower than that in RIN cells (see Table 1).

Effect of Octreotide and Gpp(NH)p on IBMX: Basal and Stimulated Cyclic AMP

The nonhydrolyzable analogue of guanine nucleotide dissociates the subunits of G_i which mediates the inhibitory action of octreotide (SMS) on adenylyl cyclase [12, 17]. We sought to determine if the difference in G_i expression in RIN cells, as shown on immunoblot analysis, was associated with a functional difference that might parallel earlier observations on the effects of pertussis toxin pretreatment on SMS actions in RIN cells [14]. Therefore, we examined the effects of SMS on basal and IBMX-stimulated cAMP accummulation.

In order to investigate whether G_i-mediated SMS action resulted in the inhibition of basal intracellular cAMP production, we incubated RIN cells with 50 nM SMS alone and in combination with 10^{-6} M Gpp. As shown in Fig. 2, SMS alone only modestly inhibited cAMP accumulation. However, SMS supplemented with Gpp exerted a more profound inhibition of cAMP accumulation (an inhibitory effect of 58%). Gpp alone elicited a response in cAMP accumulation that was not different from control (5338 ± 704 fmole/10^5 cells vs 4640 ± 587 fmole/10^5 cells).

As shown in Fig. 3, IBMX-stimulated cAMP accumulation in RIN cells under these conditions was not inhibited by 50 nM SMS. However, when 50 nM SMS was supplemented with 10^{-6} M Gpp, it significantly inhibited IBMX-stimulated cAMP (10888 ± 490 fmole/10^5 cells vs 2199 ± 356 fmole/10^5 cells).

Effect of Octreotide (SMS) Alone and in Combination with Gpp(NH)p on [^{3}H]Thymidine Incorporation into Extracted DNA

Since G proteins are known to play specific roles in transducing mitogenic signals in many endocrine tumors, and at the very least are likely to participate in signaling mechanisms for cellular proliferation [18], we investigated the effect of various concentrations of SMS on TTID in RIN cells in the presence of 1% FBS. As shown in Fig. 4, increasing concentrations of SMS (50 to 500 nM) had no effect on TTID in these rat insulinoma cells. This further supports our observations that in unstimulated cells, SMS has no inhibitory affect on RIN cell proliferation [14].

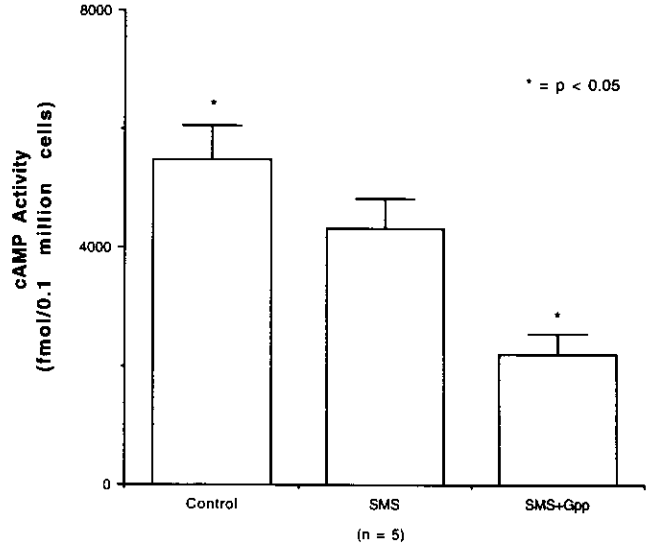

FIG. 2. The effect of 50 nM octreotide (SMS) in the absence and presence of 10^{-6} M Gpp(NH)p (Gpp) on basal intracellular cAMP accumulation in RIN cells (10^5 cells/well). The reagents were prepared in RPMI 1640 and RPMI 1640 alone was used as control. cAMP was extracted with 15% trichloroacetic acid (TCA), followed by four ether extractions. cAMP was measured by a cAMP-specific radioimmunoassay and is expressed as fmole/10^5 cells/5 min. Gpp exerted no significant effect on cAMP accumulation (see text). *$P < 0.05$ when groups were compared to each other ($n = 5$).

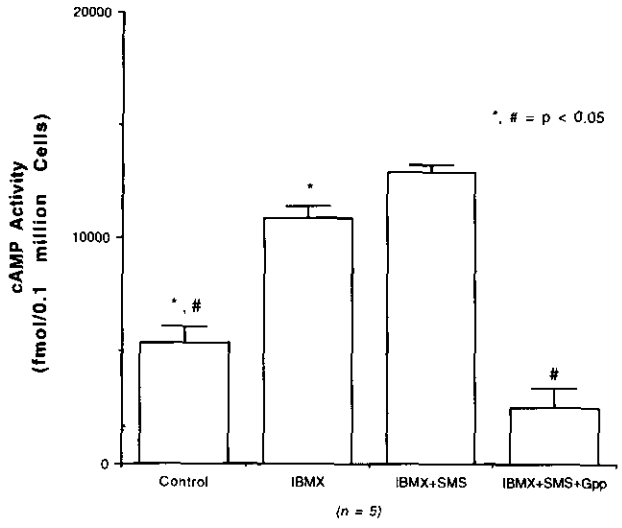

FIG. 3. The effect of 50 n*M* octreotide (SMS) in the absence and presence of 10^{-6} *M* Gpp(NH)p (Gpp) on IBMX-stimulated (10^{-4} *M* IBMX) intracellular cAMP accumulation in RIN cells (10^5 cells/well). The reagents were prepared in RPMI 1640 and RPMI 1640 alone was used as control. cAMP was extracted with 15% TCA, followed by four ether extractions. cAMP was measured by a cAMP-specific radioimmunoassay and is expressed as fmole/10^5 cells/5 min. $^{*,\#}P < 0.05$, when respective groups were compared to each other ($n = 5$).

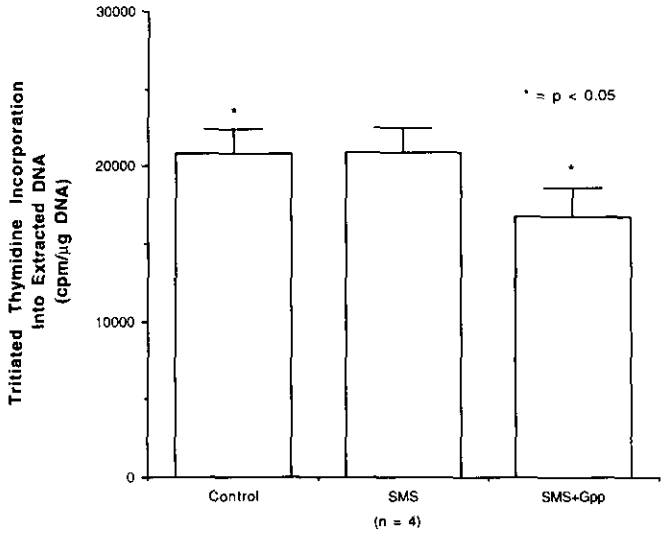

FIG. 5. The effect of 50 n*M* octreotide (SMS) on [^{3}H]thymidine incorporation into extracted DNA (TTID) in RINm5F cells in the absence and presence of 10^{-6} *M* Gpp(NH)p (Gpp) on TTID. On the ordinate (*y*-axis) TTID is indicated in cpm/5μg DNA (means ± SEM). For further details see the legend to Fig. 4. $^*P < 0.05$ when groups were compared to each other ($n = 4$).

We further investigated whether supplementation of SMS with Gpp would have any effect on TTID in RIN cells. As shown in Fig. 5, SMS supplemented with Gpp exerted a modest but significant inhibition of TTID in RIN cells (20860 ± 152 cpm/μg DNA vs 16759 ± 1912 cpm/μg DNA).

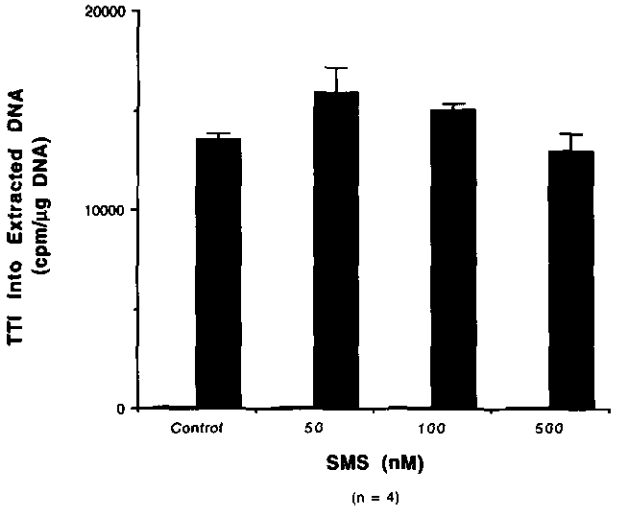

FIG. 4. The effect of increasing concentration of SMS on [^{3}H]-thymidine incorporation into extracted DNA (TTID) in RINm5F cells in the presence of 1% FBS. The control received only 1% FBS. On the ordinate (*y*-axis) TTID is indicated in cpm/μg DNA (means ± SEM). On the abscissca (*x*-axis) the concentration of octreotide (SMS) is indicated. For further details see Materials and Methods ($n = 4$).

DISCUSSION

The concept of an abnormal signal transduction system in a disease state is a relatively new one and needs to be investigated further [2, 19]. In these studies we have observed that there are differences in G_i protein expression in RINm5F cells when compared with normal rat pancreas. We identified three bands in RINm5F cells on immunoblots using an antibody specific to subtypes of inhibitory G protein. The identification of three apparent subtypes of G_i in RIN cells may be consistent with data reported in rat cDNA studies [6]. In HIT cells (a hamster insulinoma cell line), ADP-ribosylated products are shown to have molecular weights equal to 40 kDa [7]. In these studies, we used 12% polyacrylamide gels in order to improve resolution. On the basis of immunoblot analysis, it appears that G_i expression is different in RIN cells when compared to normal rat pancreas, even though we cannot exclude the possibility that acinar cell products may contribute to the degradation of G_i subunits in normal rat (mixed) pancreatic cell isolates (*Fig. 1*). We further reason that since G_i mediates cAMP inhibition, increased cAMP accumulation in RIN cells is consistent with our hypothesis that G_i is functionally altered in RIN cells (see Table 1). Additionally, somatostatin (octreotide) acts via G_i [12]. The fact that it inhibits cAMP in pancreatic acinar cells also supports this finding [20].

It has been demonstrated that low levels of insulin in experimentally induced type I diabetes mellitus leads to the loss of G_i expression in rat hepatocytes [21]. It remains to be examined whether the increased expression of G_i in RINm5F cells is related to the high levels of insulin known to be chronically secreted by these tumor cells.

Our data further documents that IBMX-stimulated cAMP accumulation in RINm5F cells is not inhibited by SMS. Somatostatin receptors have been well-documented to be present on RINm5F cells [22, 23]. In many different cell types somatostatin binds to its receptors and G_i transduces its signal (at least in part) to inhibit adenylyl cyclase and cAMP production [6, 7, 12, 13]. Data presented here (Figs. 1–3) suggests that G_i in RINm5F cells may be functionally altered and therefore may not transduce an effective signal to facilitate the inhibition of cAMP production and subsequent accumulation. This lack of an inhibitory effect by SMS is evidence for the concept of an altered expression of G_i in RIN cells.

Gpp is known to dissociate G proteins into their constituent subunits in cells that have been permeabilized. The induction of the inhibitory action of SMS in the presence of Gpp in intact cells (Figs. 2 and 3) suggests that Gpp, in such tumor cells, appears to facilitate the transmission of a more normal/functional coded SMS signal. It is likely that Gpp may have limited entry into these cells, but to an extent sufficient to affect the SMS response observed. However, this remains to be further elucidated.

Studies using normal rat pancreas have shown that somatostatin inhibits the exocrine pancreas [24]. This suggests that somatostatin may be involved in mechanisms for negative control in normal and physiological states. In addition, this mechanism of negative control mediated by somatostatin is known to be regulated by G_i in 3T3 cells and hepatocytes [10, 13]. SMS does not inhibit tritiated thymidine incorporation into extracted DNA (Fig. 4). However, in the presence of Gpp, SMS exerted an inhibitory effect on TTID in RINm5F cells. This data further supports the observations that Gpp facilitates the action of SMS in RIN cells. The requirement of SMS to be supplemented by Gpp to exert its inhibitory action further supports our findings of an altered expression of G_i in these cells. Further studies are necessary to further characterize the apparent abnormality in G_i proteins and their altered expression in this insulin-secreting, islet cell tumor cell line.

These data suggest that G_i protein expression is altered in RINm5F cells. It remains possible that a functionally altered G_i may be involved or associated with the observed inability of octreotide to reliably effect tumor growth in human insulinoma. It is likely that further study of human tumors will lead to new insights into signal transduction and the clinical implications of G protein function in insulinoma.

REFERENCES

1. Gilman, A. G. G proteins: Transducers of receptor-generated signals. *Annu. Rev. Biochem.* **56:** 615, 1987.
2. Spiegel, A. M., Gierschik, P., Levine, M. A., and Downs, R. W., Jr. Clinical implications of guanine nucleotide-binding proteins as receptor-effector couplers. *New Engl. J. Med.* **312**(1): 26, 1985.
3. Simon, M. I., Strathmann, M. P., and Gautam, N. Diversity of G proteins in signal transduction. *Science* **252:** 802, 1991.
4. Stryer, L., Bourne, H. R. G proteins: A family of signal transducers. *Annu. Rev. Cell Biol.* **2:** 391, 1986.
5. Carty, D. J., Padrell, E., Codina, J., Birnbaumer, L., Hildebrandt, J. D., and Iyengar, R. Distinct guanine nucleotide binding and release properties of the three G_i proteins. *J. Biol. Chem.* **265**(11): 6268, 1990.
6. Itoh, H., Toyama, R., Kozasa, T., Tsukamoto, T., Matsuoka, M., and Kaziro, Y. Presence of three distinct molecular species of G_i protein alpha subunit: Structure of rat cDNAs and human genomic DNAs. *J. Biol. Chem.* **263**(14): 6656, 1988.
7. Seaquist, E. R., Neal, A. R., Shoger, K. D., Walseth, T. F., and Robertson, R. P. G-proteins and hormonal inhibition of insulin secretion from HIT-T15 cells and isolated rat islets. *Diabetes* **41**(11): 1390, 1992.
8. Cormont, M., Le Marchand-Brustel, Y., Van Obberghen, E., Spiegel, A. M., and Sharp, G. W. Identification of G protein alpha-subunits in RINm5F cells and their selective interaction with galanin receptor. *Diabetes* **40**(9): 1170, 1991.
9. Seuwen, K., Magnaldo, I., and Pouyssegur, J. Serotonin stimulates DNA synthesis in fibroblasts acting through 5-HT receptors coupled to a G_i-protein. *Nature* **335:** 254, 1988.
10. Murayama, T., and Ui, M. Possible involvement of a GTP-binding protein, the substrate of islet-activating protein, in receptor-mediated signaling responsible for cell proliferation. *J. Biol. Chem.* **262**(26): 12463, 1987.
11. Igarashi, M., Strittmatter, S. M., Vartanian, T., and Fishman, M. C. Mediation by G proteins of signals that cause collapse of growth cones. *Science* **259:** 77, 1993.
12. Reisine, T., Zhang, Y. L., and Sekura, R. Pertussis toxin treatment blocks the inhibition of somatostatin and increases the stimulation by forskolin of cyclic AMP accumulation and adrenocorticotropin secretion from mouse anterior pituitary tumor cells. *J. Pharmacol. Exp. Ther.* **232**(1): 275, 1985.
13. Raper, S. E., Kothary, P. C., and Kokudo, N. Somatostatin-14 blocks the hepatotrophic effects of insulin in the rat. *J. Surg. Res.* **50**(4): 386, 1991.
14. McLeod, M. K., Kothary, P., Warnock, M., Fukuuchi, A., and Tutera, A. M. Mechanisms of somatostatin action in RINm5F cells in culture: Preliminary evidence for possible altered G protein function. *J. Surg. Res.* **53**(5): 439, 1992.
15. Goldsmith, P., Gierschik, P., Milligan, G., Unson, C. G., Vinitsky, R., Malech, H. L., and Spiegel, A. M. Antibodies directed against synthetic peptides distinguish between GTP-binding proteins in neutrophil and brain. *J. Biol. Chem.* **262:** 14683, 1987.
16. Goldsmith, P., Rossiter, K., Carter, A., Simonds, W., Unson, C. G., Vinitsky, R., and Spiegel, A. M. Identification of the GTP-

binding protein encoded by G_{i3} complementary DNA. *J. Biol. Chem.* **263:** 6476, 1988.

17. Koch, B. D., Dorflinger, L. J., and Schonbrunn, A. Pertussis toxin blocks both cyclic AMP-mediated and cyclic AMP-independent actions of somatostatin. *J. Biol. Chem.* **260:** 13138, 1985.
18. Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltoff, S. Oncogenes and signal transduction. *Cell* **64:** 281, 1991.
19. Levine, M. A. The McCune—Albright Syndrome: The why and wherefore of abnormal signal transduction. *N. Engl. J. Med.* **325:** 1738, 1991.
20. Taparel, D., Susini, C., Esteve, J. P., Diaz, J., Cazaubon, C., Vaysse, N., and Ribet, A. Somatostatin analogs: Correlation of receptor affinity with inhibition of cyclic AMP formation in pancreatic acinar cell. *Peptides* **6**(1): 109, 1985.
21. Gawler, D., Milligan, G., Spiegel, A. M., Unson, C. G., and Houslay, M. D. Abolition of the expression of inhibitory guanine nucleotide regulatory protein G_i activity in diabetes. *Nature* **327**(6119): 229, 1987.
22. Sullivan, S. J., and Schonbrunn, A. Characterization of somatostatin receptors which mediate inhibition of insulin secretion in RINm5F insulinoma cells. *Endocrinology* **121**(2): 544, 1987.
23. Sullivan, S. J., and Schonbrunn, A. The processing of receptor-bound [^{125}I Tyr^{11}]somatostatin by RINm5F insulinoma cells. *J. Biol. Chem.* **261**(8): 3571, 1986.
24. Morisset, J. Somatostatin: A potential antigrowth factor for the exocrine pancreas. *Regul. Pept.* **10**(1): 11, 1984.