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Research Article

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Glucose Activates the Carboxyl Methylation of γ Subunits of Trimeric GTP-binding Proteins in Pancreatic β Cells

Modulation In Vivo by Calcium, GTP, and Pertussis Toxin

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Abstract

The γ subunits of trimeric G-proteins (γ_1, γ_2, γ_5, and γ_7 isoforms) were found to be methylated at their carboxyl termini in normal rat islets, human islets and pure β [HIT-T15] cells. Of these, GTPγS significantly stimulated the carboxyl methylation selectively of γ_2 and γ_5 isoforms. Exposure of intact HIT cells to either of two receptor-independent agonists—a stimulatory concentration of glucose or a depolarizing concentration of K^+—resulted in a rapid (within 30 s) and sustained (at least up to 60 min) stimulation of γ subunit carboxyl methylation. Mastoparan, which directly activates G-proteins (and insulin secretion from β cells), also stimulated the carboxyl methylation of γ subunits in intact HIT cells. Stimulatory effects of glucose or K^+ were not demonstrable after removal of extracellular Ca^{2+} or depletion of intracellular GTP, implying regulatory roles for calcium fluxes and GTP; however, the methyl transferase itself was not directly activated by either. The stimulatory effects of mastoparan were resistant to removal of extracellular Ca^{2+}, implying a mechanism of action that is different from glucose or K^+ but also suggesting that dissociation of the αβγ trimer is conducive to γ subunit carboxyl methylation. Indeed, pertussis toxin also markedly attenuated the stimulatory effects of glucose, K^+ or mastoparan without altering the rise in intracellular calcium induced by glucose or K^+. Glucose-induced carboxyl methylation of γ_2 and γ_5 isoforms was vitiated by coprovision of any of three structurally different cyclooxygenase inhibitors. Conversely, exogenous PGE_2, which activates G_i and G_o in HIT cells and which thereby would dissociate α from β(γ), stimulated the carboxyl methylation of γ_2 and γ_5 isoforms and reversed the inhibition of glucose-stimulated carboxyl methylation of γ subunits elicited by cyclooxygenase inhibitors. These data indicate that γ subunits of trimeric G-proteins undergo a glucose- and calcium-regulated methylation–demethylation cycle in insulin-secreting cells, findings that may imply an important role in β cell function. Furthermore, this is the first example of the regulation of the posttranslational modification of G-protein γ subunits via nonreceptor-mediated activation mechanisms, which are apparently dependent on calcium influx and the consequent activation of phospholipases releasing arachidonic acid. (*J. Clin. Invest.* 1997. 100: 1596–1610.) Key words: pancreatic β cells • GTP-binding proteins • subunit carboxyl methylation • prostaglandin E_2 • cyclooxygenase inhibitors and insulin secretion

Introduction

Guanine nucleotide binding regulatory proteins (G-proteins)[1] play an important role in transducing a variety of extracellular signals (e.g., receptor ligands) to cellular effectors (1, 2). Two of the major classes of these proteins are the heterotrimeric G-proteins and the low molecular mass, monomeric G-proteins. Heterotrimeric G-proteins are composed of αβγ subunits; the βγ subunits form a tight complex under nondenaturing conditions. Upon activation, the GTP-bound α subunit interacts directly with various effectors (1, 2). The βγ complex is also capable of regulating several effectors, including ion channels (3), adenylyl cyclase (4), phospholipase A_2 (5), phospholipase C (6), phospholipase D (7), protein kinases (8), and phosphatidylinositol (PI)- kinases (9). Low molecular mass G-proteins also have been shown to interact, in a GTP-dependent manner, with several putative effector systems such as protein kinases (10–12), phospholipase D (13), and PI- kinases (14). Both classes of G-proteins have been identified in pancreatic β cells, including specific subcellular fractions (e.g., secretory granules; 15–22). We (15–22) and others (23–25) have presented evidence suggesting that both heterotrimeric as well as low molecular mass G-proteins may play key functional roles in the stimulus-secretion coupling of nutrient-induced insulin secretion.

Recent evidence clearly indicates that low molecular mass G-proteins and the subunits (αβγ) of trimeric G-proteins undergo posttranslational modification reactions (26, 27). For example, the α subunits of certain trimeric G-proteins undergo phosphorylation (28, 29), palmitoylation (27), and nonenzy-

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1. *Abbreviations used in this paper:* AFC, acetyl farnesyl cysteine; AGC, acetyl geranyl cysteine; $[Ca^{2+}]_e$, extracellular calcium; $[Ca^{2+}]_i$, intracellular calcium; G-protein, guanine nucleotide binding regulatory protein; GTPγS, guanosine (γ-thio)-triphosphate; MPA, mycophenolic acid; PTx, pertussis toxin; SAM, S-adenosyl methionine.

matic glycation (28). Recently, we (22) and others (30, 31) have demonstrated that the β subunit of trimeric G-proteins undergoes transient phosphorylation at a histidine residue; the phosphate in turn is transferred to nucleotide diphosphates (e.g., GDP) to yield GTP (22) using a mechanism analogous to the one mediated by nucleoside diphosphokinase (16). Recent studies by Morishita et al. (32) have indicated that γ subunits of trimeric G-proteins undergo protein kinase C–mediated phosphorylation which increases the affinity of γ subunits for α subunits. Additionally, γ subunits of trimeric G-proteins and many low molecular mass G-proteins have unique amino acid sequences at their C-terminal cysteine residues, which makes them candidates for a series of posttranslational modification reactions (e.g., isoprenylation and carboxyl methylation). In the case of low molecular mass G-proteins, such modifications increase their hydrophobicity and subsequent interaction with their membrane-bound effector proteins (30, 34). In the case of γ subunits, it has been suggested that isoprenylation and carboxyl methylation facilitate the interaction of βγ complex with their respective α subunits and receptors and/or with effectors for the βγ subunits (33). Furthermore, recent experimental evidence indicates that, unlike isoprenylation reactions, α-carboxyl methyl esterification is reversible, and therefore, potentially could be regulatory for cellular function (33).

Several low molecular mass G-proteins (e.g., Cdc42, Rac, and Rap) undergo such posttranslational modifications in pancreatic β cells (20, 21, 34, 35). Inhibition of these modifications using lovastatin (an inhibitor of isoprenylation) or AFC (an inhibitor of prenyl cysteine carboxyl methylation) interferes with nutrient-stimulated insulin secretion (34–36). Moreover, selective depletion of intracellular GTP using agents such as mycophenolic acid (MPA; 37) markedly reduced the carboxyl methylation of specific β cell G-proteins (e.g., Cdc42), glucose-induced phosphoinositide hydrolysis, and concomitant glucose-induced insulin secretion. These inhibitory effects of GTP-depletion were completely reversed by repletion of cellular GTP levels (35). However, no direct data exist regarding effects of agonists/antagonists of insulin release on trimeric G-protein function. In this study, we examined whether the γ subunits of trimeric G-proteins also undergo carboxyl methylation in pancreatic β cells, and whether such a modification step is regulated under conditions of stimulated or inhibited insulin secretion.

Methods

Materials

S-adenosyl-L-[^{3}H-methyl] methionine (73 Ci/mmol) and L-[^{3}H-methyl]methionine (72 Ci/mmol) were from New England Nuclear, Boston, MA. *N*-acetyl-S-*trans*,*trans*-farnesyl cysteine (AFC), *N*-acetyl-S-*trans*-geranyl-L-cysteine (AGC), and PGE_2 were purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA) or Cayman Chemical Co., Inc. (Ann Arbor, MI). Affinity-purified polyclonal antisera, directed against various isoforms of γ subunits and their control peptides, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The epitope corresponding to amino acid mappings at the amino termini of each of the isoforms were as follows: 2–20 (isoform 1), 2–17 (isoform 2), 2–21 (isoform 3), 2–15 (isoforms 5 and 7). MPA, ATPγS, GTP, guanosine (γ-thio)-triphosphate (GTPγS), 3-*O*-methyl glucose, actinomycin, cycloheximide, arachidonic acid methyl ester, indomethacin, aspirin, ibuprofen, protease inhibitors, phorbol myristate acetate (PMA), D600, and mastoparan were obtained from Sigma Chemical Co. (St. Louis, MO). Mastoparan 17 was purchased from Peninsula Laboratories, Inc. (Belmont, CA). Arachidonic acid was purchased from Nu Chek Prep, Inc. (Elysian, MN). PGE_2 was purchased from BIOMOL (Research Laboratories, Inc.) Lysophosphatidic acid (LPA), lysophosphatidylinositol (LPI), lysophosphatidylserine (LPhS), and lysophosphatidylethanolamine (LPE) were purchased from Serdary Research Laboratories (London, Canada). Reagents for SDS-PAGE and immunoblotting were obtained from Bio-Rad Laboratories (Hercules, CA). Pertussis toxin (PTx) was obtained from List Biologicals (Campbell, CA). Pansorbin (binding capacity of 2 mg/ml of human IgG/ml suspension) was purchased from Calbiochem Corporation (La Jolla, CA). Sinefungin was a gift from Eli Lilly Research Laboratories (Indianapolis, IN). Indo-1/AM was purchased from Molecular Probes, Inc. (Eugene, OR). RPMI 1640 medium was obtained from GIBCO BRL (Gaithersburg, MD). All other reagents were of analytical reagent grade. Purified βγ subunits (from transducin; predominantly γ_1 isoform) were generously provided by Dr. Akio Yamazaki, Kresge Eye Institute, Wayne State University (Detroit, MI).

Insulin-secreting cells

Intact pancreatic islets were isolated from male Sprague-Dawley rats as described previously (14–22, 35) and were picked manually under a stereomicroscope twice to avoid contamination by acinar cells. HIT-T15 cells (passages # 72–81) were kindly provided by Dr. Paul Robertson and Dr. Hui-Jian Zhang, University of Minnesota School of Medicine (Minneapolis, MN). INS-1 cells (passages # 45–75) were provided generously by Dr. C.B. Wollheim (University of Geneva School of Medicine, Geneva, Switzerland). They were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. For INS-1 cells, the culture medium was supplemented with 1-mM sodium pyruvate, 50-μM 2-mercaptoethanol, and 10-mM Hepes, pH 7.4. Human islets were provided generously by Dr. T. Mohanakumar, Islet Isolation Core, Washington University Medical School (St. Louis, MO).

Isolation of subcellular fractions from normal rat islets and clonal β cells

Islets, INS-1 cells or HIT cells were homogenized in 230-mM mannitol, 70-mM sucrose, and 5-mM Hepes, pH 7.4, containing 1-mM DTT and 2.5 μg/ml each of leupeptin, aprotinin, and pepstatin as described in references 14–22. In experiments involving identification of carboxyl-methylated proteins in the soluble or particulate fractions, homogenates were subjected to a single centrifugation at 105,000 *g* for 60 min to separate the total particulate fraction (pellet) from the cytosolic (soluble) fraction. The secretory granule fraction from normal rat islets was isolated by the differential centrifugation method described by us (15, 22). The purity of this fraction has been characterized using analyses of marker enzymes, insulin content, and electron microscopy (17).

Protein prenyl-cysteine carboxyl methylation assay

In cell-free preparations. Protein carboxyl methylation assays using homogenates or total particulate fraction were carried out (in a total volume of 100 μl) at 37°C for different time intervals (as described in the text) in 50-mM sodium phosphate buffer, pH 6.8, containing 1-mM EGTA and [^{3}H]S-adenosyl methionine (SAM) (100 μCi/ml) as described in (20, 35). GTPγS and other agents (e.g., AFC, sinefungin, or PGE_2) were present in their respective concentrations, as indicated in the text. The reaction was started by the addition of [^{3}H]SAM and was terminated by the addition of Laemmli buffer. Labeled proteins were then separated on SDS-PAGE (17% acrylamide). Degree of labeling was quantitated by autofluorography of dried gels (previously soaked in Fluoro-Hance autoradiography enhancer); Research Products International Corporation (Mount Prospect, IL), or ENH^{3}ANCE; DuPont-NEN (Boston, MA), or by vapor-phase equilibration assay (see below).

In intact HIT cells. For this purpose, intact HIT cells (30–40 × 10^6 cells) were detached from plates by trypsinization and left in a spinner for 3 h at 37°C. After this, cells were prelabeled with [^{3}H]me-

thionine (40–100 μCi/ml; 2 ml volume) at 37°C for 60 min. Preincubation of islets with [^{3}H]methionine was necessary to prelabel the endogenous SAM pools that are the substrates for trans-methylation reactions (20, 35). Inclusion of cycloheximide (5 μM) and actinomycin (2 μM) in the labeling medium (in order to prevent synthetic incorporation of labeled methionine into new proteins) did not significantly influence the carboxyl methylation of γ subunits. Therefore, these inhibitors were excluded from the labeling medium in all of the studies described herein. After prelabeling, cells were washed twice with an isotonic medium to remove unincorporated label, glucose (0.1 mM or 11.1 mM) or KCl (40 mM in the presence of 0.1 mM glucose) or mastoparan (30 μM plus 0.1-mM glucose) or lysophospholipids (10 μM plus 0.1-mM glucose) or TPA (200 nM) were added and the incubation was continued for additional time periods as described in the text. Other reagents (e.g., indomethacin, aspirin, ibuprofen, PGE_2, or AFC) were present in the assay medium as indicated in the text. (Stock solutions of PGE_2, indomethacin, and ibuprofen were dissolved in ethanol and diluted in the reaction mixture to achieve desired concentrations. Aspirin was dissolved in NaOH and the pH was adjusted to 7.5 with HCl after dilution in water. Appropriate diluents were included in the control tubes. In studies requiring the use of PGE_2, cyclooxygenase inhibitors, or AFC, albumin was excluded from the incubation medium to prevent binding of these reagents to albumin. Furthermore, cells were preincubated with AFC for 60 min or with cyclooxygenase inhibitors for 5 min to ensure their entry into the cells. The reaction was terminated quickly by adding ice-cold TCA (10% final; wt/vol). After incubation on ice for 10 min, tubes were centrifuged in an Eppendorf centrifuge at 8,000 rpm for 5 min at 4°C. The recovery of protein in TCA pellets was $> 96\%$; $n = 23$ determinations (35). Protein pellets were reconstituted in 100 μl of 100-mM Tris-HCl, pH 8.0, and labeled proteins were separated by SDS-PAGE and identified by fluorography as described above. Degree of carboxyl methylation of γ subunits was quantitated as base-labile [^{3}H]methanol (see below). Experimental conditions for quantitation of glucose-induced carboxyl methylation of γ subunits were identical to those described for low molecular mass G-proteins as described by us recently (35).

Farnesylcysteine methyl transferase assay

The activity of farnesylcysteine carboxyl methyl transferase was measured using AFC as substrate as described by us recently (36). The AFC methyl ester formed was extracted by adding 0.5 ml of chloroform: methanol (1:1; vol/vol) and 0.25 ml of water followed by vigorous vortex-mixing. The organic phase (125 μl) was transferred to an Eppendorf tube and dried at 40°C in a ventilation hood. After addition of 100 μl of 0.1-N NaOH containing 1% SDS, each tube was carefully placed in a vial containing scintillant and capped well. The vials were left at 37°C for 24 h to allow equilibration of the volatile [^{3}H]methanol in the scintillant before counting in a β radiation counter.

GTP-depletion and PTx-pretreatment studies

In studies addressing the effects of GTP-depletion on glucose- and K^+-induced carboxyl methylation of γ subunit in β cells, intact HIT cells (20×10^6 cells) were incubated with either diluent alone , MPA (1 μg/ml) or MPA plus guanosine (500 μM) for 3 or 6 h at 37°C. Under these conditions, MPA treatment resulted in a $> 80\%$ reduction in GTP levels, a $> 75\%$ reduction in GTP/GDP ratio, and a concomitant reduction in nutrient-induced insulin secretion from these cells (37). Coprovision of guanosine (500 μM) with MPA completely restored GTP and GTP/GDP ratios, as well as glucose-induced insulin secretion. After this preincubation, cells were washed twice with an isotonic medium, prelabeled with [^{3}H]methionine, and exposed to agonists (e.g., glucose) as described above. MPA pretreatment of HIT cells does not significantly affect the uptake of [^{3}H]methionine and subsequent incorporation into SAM pools (35).

For PTx studies, intact HIT cells were cultured overnight in the presence of diluent alone or PTx (100 ng/ml). After this, cells were washed three times with isotonic medium, and then cells were prelabeled with [^{3}H]methionine, and exposed to agonists (e.g., glucose, mastoparan, or KCl) for desired time intervals as described in the text. PTx-pretreatment did not significantly affect (not shown) uptake of [^{3}H]methionine and subsequent incorporation into SAM pools. Alternatively, PTx-catalyzed ribosylation of α subunits was quantitated using total particulate fraction (17, 38). For this purpose, PTx holoenzyme was activated at 30°C for 30 min in 10-mM Tris-HCl consisting of 10-mM DTT and 100-μM ATP. The ADP-ribosylation assay was carried out (in a total volume of 100 μl) in a reaction mixture consisting of 20-mM Tris-HCl, pH 8.0, 5-mM DTT, 100-μM GDP, 1-mM $MgCl_2$, 10-mM thymidine, and 2 μg/ml of activated PTx. The reaction was initiated by the addition of [^{32}P]NAD (10 μM; 0.5 μCi per tube) and was continued for 1 h at 37°C that was terminated by the addition of Laemmli sample buffer. Labeled proteins were separated by SDS-PAGE and identified by autoradiography of dried gels.

Vapor-phase equilibration assay

The α-carboxyl methyl groups on prenyl cysteine residues of modified γ subunits are base-labile. To demonstrate that such sites are modified on the γ subunits in insulin-secreting cells, [^{3}H]methyl esters were quantitated by vapor-phase equilibration assay, as described in references 20 and 35. Briefly, individual lanes of dried gels were cut into 5-mm slices and were placed in 1.5-ml Eppendorf centrifuge tubes (without caps) containing 500 μl of 1-N NaOH. Tubes were placed in 20-ml scintillation vials containing 5-ml scintillation fluid (Ultima Gold; Packard Instrument Co., Meriden, CT). The vials were then capped and left at 37°C overnight to maximize the base-labile release of [^{3}H]methanol due to hydrolysis of methyl esters, and the radioactivity was determined by scintillation spectrometry.

Extraction and immunological identification of various isoforms of γ subunits

γ subunits from homogenates of HIT cells, INS-1 cells and normal rat islets homogenates were extracted with 20-mM Tris, pH 8.0, containing 1-mM DTT, 1-mM EDTA, and 1% cholate as described in reference 39. After extraction, proteins were separated by SDS-PAGE (17%) and blotted onto nitrocellulose membranes that were incubated with antisera (1:500 dilution) against various γ isoforms; protein bands were detected by chemiluminescence using a kit purchased from Bio-Rad Laboratories. Molecular masses of proteins were determined using prestained authentic molecular mass standards (Bio Rad Laboratories) as well as purified γ subunits (transducin).

Immunoprecipitation of methylated γ subunits

Intact HIT cells were prelabeled with [^{3}H]methionine as described above. After this, cells were rapidly washed using isotonic medium and resuspended in the same medium and aliquoted (200 μl per tube) into 1.5-ml Eppendorf tubes. Cells were incubated then with different agonists (e.g., glucose, KCl, or mastoparan) for different time intervals as indicated in the text. After the reaction, the tubes were rapidly centrifuged in an Eppendorf centrifuge at 4°C (1000 rpm for 3 min). Medium was aspirated quickly using a pasteur pipette and 500 μl of cholate extraction medium (consisting of 20-mM Hepes, pH 7.4, 1% sodium cholate, 2-mM $MgCl_2$, 1 mM each of DTT, EDTA, benzamidine, and PMSF, 10 μg/ml each of aprotinin, leupeptin, and pepstatin) was added. Contents of the tubes were sonicated (3×10 s) using a bath sonicator, and the tubes were shaken gently at 4°C for 2 h. Then, cholate extracts were incubated with preimmune serum or antisera (1:500 dilution) directed against various isoforms of γ subunits overnight at 4°C. Immune-complexes were captured using Pansorbin, and separated by centrifugation. Pellets were washed three times with 20-mM Tris-HCl, pH 7.4, containing 150-mM NaCl, and base-labile [^{3}H]methanol released in the immunoprecipitates was quantitated by scintillation spectrometry as described above. All values were adjusted by subtracting nonspecific [^{3}H]methanol released in immunoprecipitates using preimmune serum.

Specificity of Gγ antisera (used in immunoblotting and immuno-

precipitation studies) was also verified by immunoneutralization. This was carried out by preincubating antisera with ten-fold excess (wt/wt) of control peptides in PBS for 2 h at 25°C before incubation with nitrocellulose membranes. Additional control studies indicated that purified βγ subunits from transducin (predominantly γ_1 isoform) reacted positively only with antiserum directed against γ_1 isoform. It was also observed that > 80% of the carboxyl-methylated γ_1 subunit catalyzed by HIT cell homogenates was precipitated under our experimental conditions (14% variance; $n = 8$).

Quantitation of demethylation of γ subunits

Proteins in the homogenates of normal rat islets were labeled (in a total volume of 400 μl) with [^{3}H]SAM for 60 min as described above. After this, additional carboxyl methylation of γ subunits was inhibited by the addition of 1-mM sinefungin, which is a structural analog of S-adenosyl homocysteine and an inhibitor of methylation reactions (35, 40). At this concentration, additional carboxyl methylation of γ subunit is inhibited by 80%; see below. The reaction was continued for an additional 5 h at 37°C, and aliquots of 75 μl were drawn at different time intervals as indicated in the text. The degree of residual labeling of γ subunits was determined by the vapor-phase equlibration assay after SDS-PAGE as described above.

Measurement of intracellular calcium ($[Ca^{2+}]_i$)

Cytosolic free calcium concentrations ($[Ca^{2+}]_i$) were determined as described previously (41). To test the effects of PTx pretreatment on glucose- and K^+-stimulated increases in cytosolic-free calcium, HIT cells were treated with PTx (100 ng/ml) overnight in culture medium and during 3 h recovery in spinner medium after detachment. Subsequently, cells were loaded with indo-1/acetoxymethyl ester (1 μM) for 30 min at 37°C. About 2×10^6 cells (in 2-ml KRB-Hepes buffer; glucose free) were transferred to a cuvette. After equilibration at 37°C for 20 min, various stimulators (e.g., glucose or K^+) were added. Fluorescence was recorded using a Perkin Elmer spectrofluorometer (model LS50B), with excitation and emission wavelengths of 355 nm and 405 nm, respectively. $[Ca^{2+}]_i$ was determined based on the calibration method described by Grynkiewicz et al. (42).

Other methods

Protein concentration was quantitated colorimetrically using a dye-binding method described by Bradford (43). Data are expressed as mean±SEM with n representing the number of determinations. Statistical analyses were performed by nonpaired t tests or the Mann-Whitney test, as appropriate.

Results

Carboxyl methylation and demethylation of γ subunits in HIT cell homogenates: effects of GTPγS and AFC. The carboxyl methylation of a protein in the M_r region of 5–7 kD was quantitated by base-labile methanol assay; this protein comigrated with purified transducin γ subunit (not shown). Carboxyl methylation of this protein was time-dependent and reached saturation by 150 min; it was dependent upon HIT cell protein concentration, being linear up to 60 μg protein per assay. Most (> 85%) of the activity was demonstrable in the total particulate fraction (additional data not shown). These data indicate that both the methyl transferase and its methyl acceptor protein substrate (i.e., γ subunits) are associated, to a large degree, with the particulate fraction, compatible with our recent observations that prenyl cysteine methyl transferase activity is predominantly membrane-associated in normal rat islets as well as in pure β (HIT-T15 or INS-1) cells (36).

At least 11–12 different isoforms of γ subunits of trimeric G-proteins have been identified in mammalian cells thus far (44). To determine which isoforms are present in β cells, HIT or INS-1 cell homogenates were examined by immunoblotting using specific antisera directed against various isoforms of γ subunits. γ_1, γ_2, γ_5, and γ_7 isoforms were readily detected in both HIT or INS-1 cells; however, under our current experimental conditions, the γ_3 isoform was undetectable in these preparations (Fig. 1). The effect of GTPγS, a nonhydrolyzable analog of GTP, on the carboxyl methylation of individual isoforms of γ subunits was investigated in the particulate fraction after immunoprecipitation. Of all the four isoforms studied, GTPγS (100 μM) specifically stimulated the carboxyl methylation of only γ_2 and γ_5 (Fig. 2). A modest (+22%), but not significant ($P > 0.7$; degrees of freedom = 10), stimulation by GTPγS of the carboxyl methylation of γ_7 isoform was also demonstrable, whereas methylation of γ_1 was unaffected by GTPγS (Fig. 2).

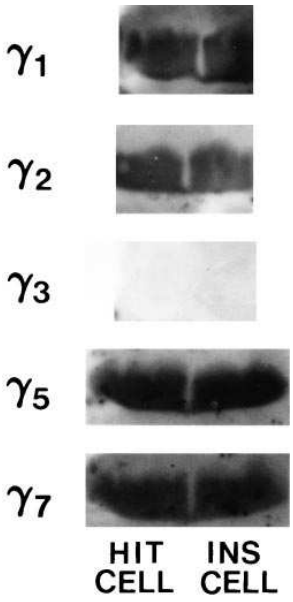

Figure 1. Immunologic detection of various isoforms of γ subunits in HIT cell and INS-1 cell homogenates. HIT cell and INS-1 cell homogenate proteins were extracted in cholate (1%) containing media (see Methods) and separated by SDS-PAGE (17% gels). After this, proteins (4–8 kD) were transferred onto nitrocellulose membranes and probed with specific antisera directed against individual isoforms of γ subunits, and antigen–antibody complexes were detected by chemiluminescence method. Specificity of these antisera was also examined by peptide neutralization that was carried out by preincubating antisera with 10-fold excess (wt/wt) of their respective control peptides in PBS for 2 h at 25°C before incubation with nitrocellulose blots. Data are representative of 2–3 blots for each isoform in each of the cell lines.

Both the α-carboxyl methyl esters at C-terminal cysteine residues (35) and at other amino acids at the C-terminal (e.g., leucine, as in the case of protein phosphatase 2A catalytic subunit) are base-labile (40). Therefore, we investigated the effect of AFC, a substrate for (and selective competitive inhibitor of) prenyl cysteine methyl transferase (20, 35), but not leucine methyl transferase (40). AFC (100 μM) completely inhibited the stimulatory effect of GTPγS (Table I) on the carboxyl methylation of γ_2 and γ_5 subunits in HIT cell homogenates. In contrast, acetyl geranyl cysteine (AGC; 100 μM), a 10 carbon analog of AFC that is neither a substrate for (nor an inhibitor of) the methyl transferases (20, 35), had no demonstrable effect on the carboxyl methylation of γ_2 subunit (98% of control; $n = 2$ determinations). We also observed that γ subunit was carboxyl methylated in human islet homogenates (not shown); AFC (100 μM) modestly, but significantly, reduced the carboxyl methylation of this protein (22±1.2 fmol of ^{3}H-methanol released in the absence of AFC versus 16.85±2 fmol of ^{3}H-methanol released in its presence; $n = 3$ determinations each; $P = 0.025$). Furthermore, in preliminary studies, we also observed that a protein in the M_r region of 5–7 kD was also car-

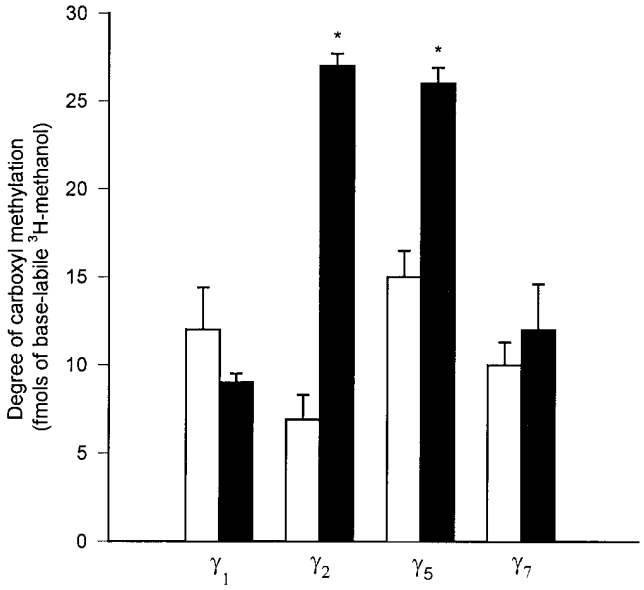

Figure 2. Stimulation by GTPγS of the carboxyl methylation of γ subunit isoforms in HIT cell homogenates. HIT cell homogenates were incubated with [^{3}H]SAM for 120 min in the absence or presence of GTPγS (100 μM). After the incubation, proteins were extracted using cholate-containing media, and individual isoforms of γ subunits were immunoprecipitated (see Methods) and the degree of carboxyl methylation in the immunoprecipitates was quantitated by vapor-phase equilibration assay. Data are mean±SEM from 4–11 individual immunoprecipitations in each case. Data are expressed as fmols of ^{3}H-methanol released in the absence (*open bars*) or presence (*closed bars*) of GTPγS. GTPγS significantly stimulated the carboxyl methylation of both γ_2 and γ_5 isoforms (*$P < 0.001$).

boxyl-methylated in the secretory granule fraction from normal rat islets, although the limited amount of protein obtainable from the secretory granule fractions precluded definitive immunologic identification of this protein. Together, these data suggest that the carboxyl-methylated amino acid residue on the γ subunit is probably cysteine.

To examine whether the prenyl cysteine methyl transferase activity endogenous to HIT cell can methylate exogenously purified γ subunits, purified βγ subunits were incubated in different concentrations (0–900 ng) with HIT cell homogenates and [^{3}H]SAM. These data demonstrate a concentration-dependent increase in the carboxyl methylation of γ subunit that was linear up to 600 ng per assay. GTPγS (100 μM) did not affect (not shown) the carboxyl methylation of purified transducin γ subunit (+10%), compatible with lack of a clear effect of GTP on the carboxyl methylation of endogenous γ_1 subunits (Fig. 2). However, AFC (100 μM) markedly (−71%) reduced the carboxyl methylation of purified γ subunit (additional data not shown) as did 1-mM sinefungin (a structural analog of S-adenosyl homocysteine and an inhibitor of protein methyl transferases; −80%; mean of two determinations). These data suggest that βγ subunits can be carboxyl-methylated without the necessity for incorporation into αβγ trimer. Recently Tan and Rando (45) described the presence of a demethylating enzyme (i.e., carboxyl methyl esterase) in rod outer segments that catalyzes the hydrolysis of methyl ester groups from the carboxyl methylated cysteine residues of low molecular mass G-proteins as well as from γ subunits of tri-

Table I. Inhibition by AFC of the GTPγS-stimulated Carboxyl Methylation of γ_2 and γ_5 Isoforms in HIT Cell Homogenates

	Degree of carboxyl methylation (fmol of base-labile [^{3}H]methanol released)	
Condition(s)	Isoform 2	Isoform 5
None	13±0.7	14±0.6
GTPγS	31±1	29±1.7
GTPγS plus AFC	10±0.6	8±0.6

HIT cell proteins were carboxyl methylated using [^{3}H]SAM in the presence of diluent alone or GTPγS (100 μM) or GTPγS plus AFC (100 μM each) for 120 min at 37°C. γ_2 and γ_5 isoforms were immunoprecipitated (see Methods) and the degree of carboxyl methylation in the immunoprecipitates was quantitated by vapor-phase equilibration assay. Data are mean±SEM from 4–6 determinations in each case. GTPγS significantly stimulated ($P < 0.001$) the carboxyl methylation of each isoform and this effect was completely inhibited by AFC.

meric G-proteins. Herein, we investigated whether γ subunit demethylating esterase activity is also present in β cells. γ subunits were nearly totally (−86%) demethylated after 5 h of incubation at 37°C; half-maximal demethylation occurred by ~ 3.5–4 h (Fig. 3). Inclusion of ebelactone (500 μM), an es-

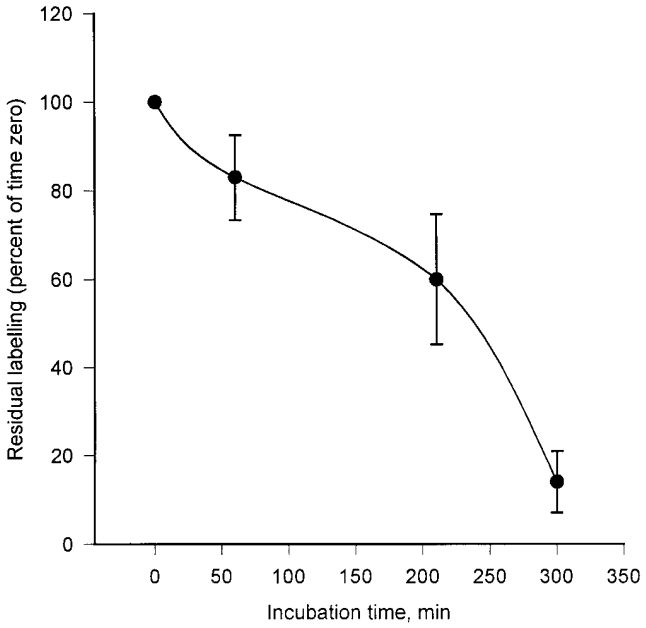

Figure 3. Time course of demethylation of γ subunit in normal rat islet homogenates. Rat islet homogenate proteins (800-μg protein) were carboxyl methylated (total volume of 400 μl) in the presence of [^{3}H]SAM for 60 min at 37°C. After the reaction, additional carboxyl methylation of γ subunits was inhibited by addition of 1-mM sinefungin. The reaction was continued for an additional 5 h at 37°C, and aliquots of the reaction mixture (75 μl) were drawn at different time intervals as indicated in the figure. Proteins were separated by SDS-PAGE (17% acrylamide), and the degree of residual labeling was determined by base-labile methanol assay. Data are mean±SEM from four individual determinations at each point and expressed as residual labeling at each time point. Labeling at zero time point was taken as 100%.

terase inhibitor (40, 45), markedly reduced the demethylation of γ subunit (additional data not shown), suggesting that the later is mediated by a demethylating esterase similar to the one described by Tan and Rando recently (45).

Regulation of the carboxyl methylation of γ subunits in intact HIT cells. The carboxyl methylation of γ subunits was then studied in the absence (0.1-mM glucose) or presence of a stimulatory concentration (11.1 mM) of glucose, the major physiologic stimulus for insulin release. Intact HIT cells were used since glucose is not a receptor agonist and its effects require its metabolism in intact cells. Base-labile methanol was quantitated in gel slices in 5–7 kD range (i.e., methylation of the total G_γ pool). High glucose (11.1 mM) stimulated the carboxyl methylation of γ subunits (Fig. 4); this effect was demonstrable as early as 30 s (1.4±0.09-fold; $n = 10$ experiments) and persisted even after 60 min of stimulation (1.5±0.07-fold; $n = 3$ experiments). 3-*O*-methyl glucose, an analog of glucose that is transported like glucose but not metabolized, had no significant effect (105±3% of control after 2 min of exposure; $n = 4$ determinations; $P = 0.4$). The stimulatory effect of glucose was also demonstrable in intact normal rat islets (3.31±0.15 fmol of ^{3}H-methanol released in the presence of 3.3-mM glucose versus 4.3±0.19 fmol ^{3}H-methanol released in the presence of 16.7-mM glucose; $n = 3$ determinations; $P = 0.03$). We also observed that 40-mM KCl also consistently stimulated the carboxyl methylation of (the total pool of) γ subunits; these stimulatory effects (1.55-fold) persisted for up to 30 min of incubation in a manner similar to glucose's effects (additional data not shown).

Table II. Role of Extracellular Calcium on Glucose or K^+-stimulated Carboxyl Methylation of γ Subunits in Intact HIT Cells

Conditions	Degree of methylation (fmol of base-labile methanol)	*P* versus 0.1-mM glucose
Glucose (0.1 mM)	5.6±0.21	—
Glucose (11.1 mM)	11.5±3.2	0.030
Glucose (11.1 mM) + EGTA (5 mM)	6.9±0.8*	0.100
KCl (40 mM)	8.7±0.28	0.003
KCl (40 mM) + EGTA (5 mM)	7.0±0.02**	0.180

Intact HIT cells were prelabeled with [^{3}H]methionine for 1 h as described in the text. After this, cells were washed twice with an isotonic medium and were exposed to glucose or K^+ as indicated in the table. Where stated, cells were preincubated with 5-mM EGTA to chelate extracellular calcium before exposure to glucose or KCl. Data are expressed as fmol of base-labile [^{3}H]methanol released and represented as mean±SEM from three individual determinations. Removal of extracellular calcium markedly reduced the ability of glucose or K^+ to stimulate the carboxyl methylation of γ subunits in intact HIT cells (*$P = 0.005$ versus high glucose alone and **$P = 0.005$ versus 40-mM KCl).

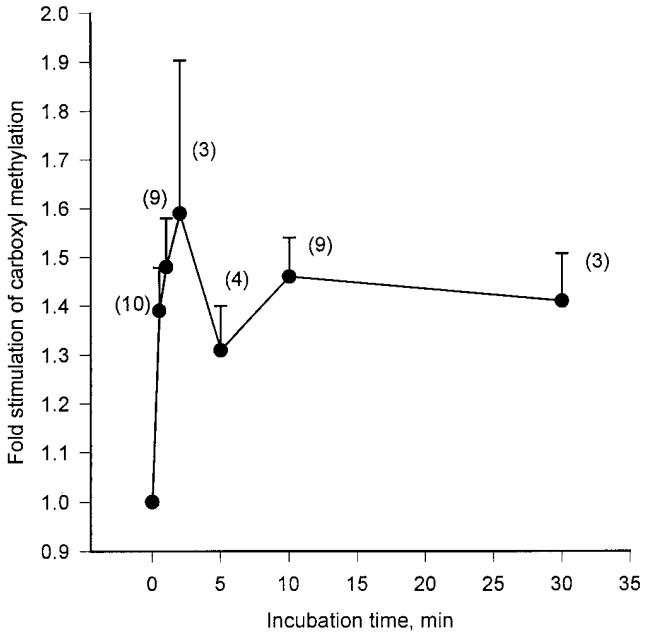

Figure 4. Stimulation by glucose of the carboxyl methylation of γ subunits in intact HIT cells. Intact HIT cells were labeled with [^{3}H]methionine for 1 h as described in Methods. After two rapid washes with isotonic medium, cells were incubated with 0.1- or 11.1-mM glucose for different time intervals as indicated in the figure. Reaction was terminated by the addition of 10% TCA (wt/vol; final), proteins were separated by SDS-PAGE (17% acrylamide) and the degree of carboxyl methylation of the γ subunit was quantitated by base-labile methanol release. Data are expressed as fold stimulation by 11.1-mM glucose of the carboxyl methylation of γ subunits (total pool) over basal methylation (i.e., observed in the presence of 0.1-mM glucose at the same time points). The basal carboxyl methylation represented 5–6 fmol of base-labile ^{3}H-methanol. Data are mean±SEM from the number of individual determinations indicated in the parentheses at each time point.

Mastoparan, a tetradecapeptide from wasp venom, which stimulates G-protein function in a receptor-independent manner (46), also stimulates insulin secretion in a concentration-dependent manner from normal rat islets (20) as well as from clonal β cells (47). Mastoparan (30 μM) stimulated the carboxyl methylation of γ subunits (181±24% of control cells; degrees of freedom = 25; $P = 0.0001$) whereas Mastoparan 17, an inactive structural analog of mastoparan (20), failed to stimulate carboxyl methylation (+22±7%; $n = 4$ determinations; P = not significant versus control).

Glucose- and K^+-induced insulin secretion from pancreatic β cells is dependent upon extracellular calcium (48). In order to examine whether extracellular Ca^{2+} fluxes contribute to the glucose- and potassium-induced carboxyl methylation of γ subunits, HIT cells were preincubated for 10 min with EGTA (5 mM) to chelate extracellular calcium and then the effects of glucose and potassium on the carboxyl methylation of γ subunits were tested. Removal of calcium abolished the stimulatory effects of glucose and potassium, implying a key regulatory role(s) for extracellular calcium in this phenomenon (Table II). Furthermore, inclusion of 7-mM $CaCl_2$ in the incubation medium (thus approximating 2-mM free calcium) completely reversed the inhibitory effects of EGTA on glucose-induced carboxyl methylation (i.e., 187±12% of control; $n = 3$ determinations; P value versus glucose plus EGTA alone = 0.006). Interestingly, unlike glucose or potassium, stimulation by mastoparan of the carboxyl methylation of γ subunits was resistant to removal of extracellular calcium (i.e., 194±3% of control in the presence of 5-mM EGTA in the incubation me-

dium versus 181±24% in the presence of mastoparan alone; n = 3 determinations).

Inhibition by PTx-pretreatment of glucose-, potassium-, or mastoparan-sensitive carboxyl methylation of γ subunits. PTx induces ADP-ribosylation of some trimeric G-proteins (G_i, G_o, and G_t) resulting in uncoupling of the αβγ trimer from the cognate receptor (1); PTx also interferes with mastoparan-induced insulin secretion from normal islets (20) and certain clonal β cells (47). PTx-sensitive trimeric G-proteins are present in human and rodent β cells (for review see references 19, 23, and 25). Therefore, we investigated whether PTx pretreatment interferes with carboxyl methylation of γ subunits in intact HIT cells. After preincubation with PTx, no ribosylation was detectable in the membrane fraction, indicating near complete ribosylation of endogenous PTx-sensitive α subunits under our experimental conditions (additional data not shown). PTx abrogated the ability of glucose (Fig. 5), mastoparan or potassium (Table III) to augment the carboxyl methylation of total γ subunit pool. Interestingly, after PTx-pretreatment glucose actually reduced the carboxyl methylation of (total pool of) γ subunit within 30 s of exposure (Fig. 5). This may be because of its specific inhibitory effects on certain PTx-insensitive isoform(s) of γ subunits (see below). It is noteworthy that, in contrast, PTx-treatment did not affect the ability of glucose to stimulate the carboxyl methylation of Cdc42, a low molecular mass G-protein, which we have recently implicated in nutrient-induced insulin secretion (35). The degree of stimulation by glucose of the carboxyl methylation of Cdc42 in the control and Ptx-treated cells was 1.87-fold and 1.62-fold, respectively (mean of three determinations; additional data not shown). These data suggest that the PTx effects on glucose-stimulated carboxyl methylation of γ subunits are distal to Cdc42 activation by glucose (see below).

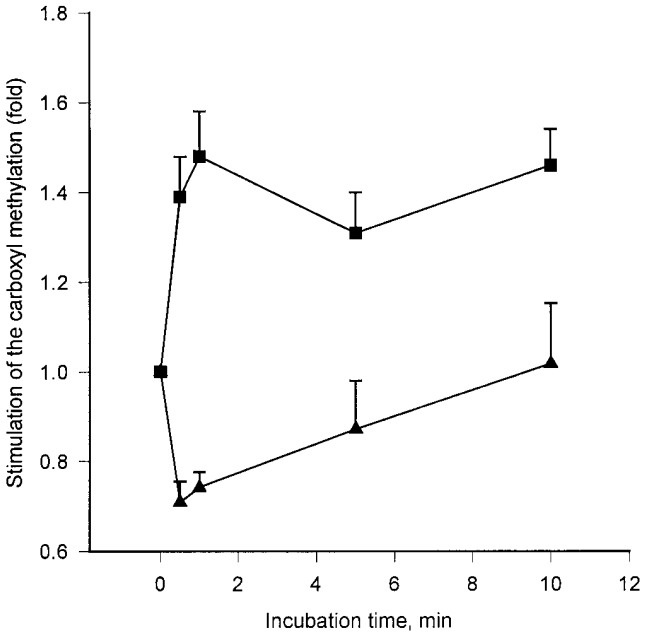

Figure 5. Inability of glucose to stimulate the carboxyl methylation of γ subunits in intact HIT cells pretreated with PTx. Intact HIT cells were cultured overnight in the presence of diluent alone (■) or PTx (100 ng/ml; ▲). After two rapid washings with isotonic medium, cells were prelabeled with [^{3}H]methionine for 1 h as described in Methods. After this, cells were exposed to 0.1- or 11.1-mM glucose for different intervals as indicated in the figure. Labeled proteins were separated by SDS-PAGE and carboxyl methylation of γ subunits was quantitated by vapor-phase equilibration assay. Basal methylation at time zero in the presence of 0.1-mM glucose corresponded to a base-labile [^{3}H]methanol release equivalent to 6 fmol. Data are mean±SEM from number of determinations indicated in parentheses.

Table III. Effect of PTx Pretreatment on K^+ and Mastoparan-stimulated Carboxyl Methylation of γ Subunit in Intact HIT Cells

Conditions	Control cells	*P* versus respective control	PTx-treated cells	*P* versus 0.1-mM glucose
Glucose (0.1 mM)	6.2±0.4	—	7.5±0.74	Not significant
KCl (40 mM)	10.35±0.5	0.014	5.95±0.15	0.035
Mastoparan (30 μM)	10.98±1.5	0.018	5.9±0.523	0.001

Intact HIT cells were cultured overnight in the presence of diluent alone to PTx (100 ng/ml). After this, cells were washed with an isotonic medium and were prelabeled with [^{3}H]methionine as described in the text. After two brief washes, they were exposed to KCl (40 mM) or mastoparan (30 μM) for 2 min at 37°C. The reaction was terminated by the addition of 10% TCA and labeled proteins were separated by SDS-PAGE. Degree of methylation was quantitated by vapor-phase equilibration assay. Data are expressed as fmol of base-labile [^{3}H]methanol released and represented as mean±SEM from 3–5 determinations. These data suggest that pretreatment of intact HIT cells with PTx results in attenuation of stimulation of the γ subunit carboxyl methylation by K^+ and mastoparan.

Since the data above indicated a requirement for calcium entry in glucose- and potassium-stimulated carboxyl methylation of γ subunits, we examined whether the attenuated responses to glucose and potassium in PTx-treated cells could be attributed to changes in calcium fluxes. The results indicated that PTx-pretreatment (100 ng/ml; overnight) of HIT cells induced no major changes in glucose- or potassium-induced cytosolic free calcium concentrations (Table IV).

Identification of methylated γ subunit isoforms in intact β cells: effects of glucose, mastoparan, or potassium. To determine which of the γ isoforms is stimulated by these agonists, meth-

Table IV. Glucose- and K^+-stimulated Cytosolic-free Calcium Concentrations in Intact HIT Cells: Effect of PTx Pretreatment of HIT Cells

Condition(s)	$[Ca^{2+}]_i$ in control cells (nM)	$[Ca^{2+}]_i$ in PTx-treated cells (nM)
Basal	154±3 (10)	155±6 (8)
Glucose (10 mM)	272±12 (5)*	290±15 (5)*
KCl (40 mM)	584±54 (5)*	555±41 (3)*

HIT cells were treated with diluent alone or PTx (100 ng/ml) overnight in culture (see Methods) and subsequently were loaded with Indo and stimulated with glucose or KCl as indicated in the table. Fluorescence was measured using a Perkin Elmer spectrofluorometer. Data are mean±SEM from number of observations as indicated in the parentheses. PTx treatment did not affect the ability of glucose or KCl to raise $[Ca^{2+}]_i$ concentrations. *$P < 0.001$.

ylated γ subunits were immunoprecipitated using specific antisera directed against various isoforms, or using preimmune serum as control (for quantitation of nonspecific precipitation). Under basal conditions, all the four isoforms underwent carboxyl methylation, albeit to varying degrees; however, the carboxyl methylation of only three of Gγ isoforms was stimulated by glucose, with the following rank order: γ_5 (+99%; $P < 0.001$) > γ_2 (+56%; $P < 0.06$) > γ_1 (+24%; $P = 0.04$). Interestingly, the carboxyl methylation of γ_7 isoform was inhibited (−32%; $P = 0.001$) by glucose. In contrast, KCl (40 mM) stimulated the carboxyl methylation of all four isoforms, albeit to varying degrees (γ_5 [63%] > γ_1 [41%] = γ_2 [40%] > γ_7 [14%]). Mastoparan (30 μM) stimulated the carboxyl methylation of only γ_2 (+184%) and γ_5 (+64%) isoforms, but inhibited the carboxyl methylation of γ_1 (−62%; mean of two experiments). These data imply a differential regulation of the carboxyl methylation of specific γ subunits by various β cell agonists, but demonstrate that all three PTx-sensitive agonists (i.e., glucose, potassium, and mastoparan) promote the carboxyl methylation of both γ_2 and γ_5.

Intracellular GTP is required for glucose-mediated stimulation of the carboxyl methylation of γ subunits. Recently, we have shown (35) that glucose-stimulated carboxyl methylation of Cdc42 is reduced markedly by depletion of intraislet GTP using MPA. MPA pretreatment induced a marked reduction in the ability of either glucose or potassium to stimulate the carboxyl methylation of the γ subunit pool (Table V). These data support our data obtained using cell-free preparations in which exogenously added GTPγS stimulated the carboxyl methylation of γ subunits. Additional immunoprecipitation studies revealed that glucose failed to augment the carboxyl methylation of γ_2 (71±5% of control; $n = 4$ determinations) and γ_5 (77±6% of control; $n = 4$ determinations) isoforms in HIT cells pretreated with MPA for 3 h. Under these conditions, while intracellular GTP, GTP/GDP ratio, and nutrient-induced insulin secretion were markedly reduced (Meredith, M., G. Li, and S.A. Metz; manuscript submitted for publication), the peak intracellular calcium concentration achieved during glucose-stimulation remained unchanged (Li, G., and S.A. Metz, unpublished obseervations). These data indicate that impairment of glucose-induced carboxyl methylation of γ subunits may largely be due to the effects of availability of intracellular GTP on the activation of one (or more) G-protein(s) and not due to activation of calcium fluxes. In support of this formulation, we also observed that coprovision of guanosine (500 μM) along with MPA (for 3 h) which restores GTP, GTP/GDP ratio, and nutrient-induced insulin secretion to normal (Meredith, M., G. Li, and S.A. Metz; manuscript submitted for publication), also restored the ability of glucose to stimulate the carboxyl methylation of γ_2 (233±21% of control; $n = 3$ determinations) and γ_5 (197±7% of control; $n = 3$ determinations) isoforms.

Effects of PGE_2 and cyclooxygenase inhibitors on the carboxyl methylation of γ subunits. The data above present a novel situation, in that nonreceptor-mediated agonists promoted the carboxyl methylation of γ subunits in a fashion sensitive to calcium and GTP; the sensitivity to PTx implies that one or more trimeric G-proteins (e.g., G_i and G_o) are involved. Such G-proteins classically are activated by receptor-mediated agonists, usually in a calcium-independent fashion. Therefore, we considered the possibility that glucose could act via calcium- and GTP-sensitive phospholipases generating PGE_2, a known receptor agonist for G_i/G_o in HIT cells (24, 25). Several recent studies have demonstrated the presence of PTx-sensitive, high affinity GTPase activities in the membrane and secretory granule fractions derived from normal rat islets, human islets (15, 22), and clonal β cell preparations (48), which were stimulated by receptor agonists (e.g., PGE_2 and somatostatin; 15, 22) or by nonreceptor agonists such as mastoparan (15, 48). Incubation of HIT cell homogenates with PGE_2 (10 μM) markedly stimulated the carboxyl methylation of γ_2 and γ_5 isoforms (Table VI). The degree of stimulation by PGE_2 of each of these isoforms was comparable to that induced by GTPγS. Moreover, coprovision of GTPγS (100 μM) along with PGE_2 (10 μM) did not further stimulate the carboxyl methylation of either of the two isoforms (Table VI) implying a common pathway of action. Since PGE_2 stimulated the carboxyl methylation of γ_2 and γ_5 isoforms in HIT cell homogenates (Table VI), we also verified effects of PGE_2 on the carboxyl methylation of these

Table V. Effect of GTP Depletion on Glucose and K^+-stimulated Carboxyl Methylation of γ Subunits in Intact HIT Cells

Conditions	Control cells	*P* versus 0.1-mM glucose	MPA-treated cells	*P* versus respective control
0.1-mM glucose	5.8±0.25	—	5.77±0.30	NS
11.1-mM glucose	10.4±0.48	0.0001	6.34±0.27	0.005
40-mM KCl	9.4±0.45	0.034	6.62±0.3	0.001

Intact HIT cells were cultured in the presence of MPA (1 μg/ml) for 6 h as described in the text. After this, cells were prelabeled with [^{3}H]methionine and then exposed to glucose (0.1 or 11.1 mM) or KCl (40 mM), and the reaction was terminated by the addition of 10% TCA (final; wt/vol). Labeled proteins were separated by SDS-PAGE and degree of the carboxyl methylation was quantitated by vapor phase equilibration assay. Data are expressed as fmol of base-labile [^{3}H]methanol released and represented as mean±SEM from 4–5 determinations. GTP depletion markedly reduced the ability of glucose or K^+ to stimulate the carboxyl methylation of γ subunits in intact HIT cells.

Table VI. Stimulation by GTPγS and PGE_2 of the Carboxyl Methylation of γ_2 and γ_5 Isoforms in HIT Cell Homogenates

	Degree of carboxyl methylation (fmol of base-labile [^{3}H]methanol)	
Condition(s)	Isoform 2	Isoform 5
None	7±0.7	13±1.4
PGE_2, 10 μM	35±7*	42±2.6*
GTPγS, 100 μM	30±4*	37±2*
PGE_2 plus GTPγS	43±0.5*	40±4*

HIT cell homogenate proteins were incubated in the presence of PGE_2 (10 μM) alone, GTPγS (100 μM) alone, a combination of PGE_2 and GTPγS as indicated in the table. Proteins were carboxyl methylated using [^{3}H]SAM as described in Methods. After incubation, γ_2 and γ_5 isoforms were immunoprecipitated and their carboxyl methylation monitored in the immunoprecipitates by vapor-phase equilibration assay. Data are mean±SEM from four determinations in each condition. *P = 0.001 versus control.

proteins in intact HIT cells. PGE_2 (10 μM; 5 min exposure) significantly stimulated the carboxyl methylation of γ_2 (129±9%; $P = 0.037$ versus control; df = 10) and γ_5 (132±8%; $P = 0.003$ versus control; df = 12) isoforms. Interestingly, PGE_2-mediated stimulation of the carboxyl methylation of γ subunits (e.g., γ_2 isoform) in intact HIT cells could withstand a major reduction in intracellular GTP content, since comparable degrees of stimulation by PGE_2 (10 μM) of the carboxyl methylation of γ_2 isoform were demonstrable in the control and GTP-depleted cells (i.e., +113±8% in control cells versus +89±6% in GTP-depleted cells; $n = 3$ determinations in each case), suggesting that intracellular GTP may be required at a step proximal to PGE_2 synthesis (e.g., phospholipase activation; see below).

To examine whether glucose's effects are mediated by endogenous generation of PGE_2, the carboxyl methylation of individual isoforms was monitored in HIT cells incubated with glucose (11.1 mM) or glucose (11.1 mM) plus either of three structurally different inhibitors of conversion of arachidonic acid (AA) into prostaglandins via the cyclooxygenase pathway. Aspirin (1 mM), ibuprofen (120 μM), or indomethacin (2 μM) completely inhibited glucose-stimulated carboxyl methylation of γ_2 (Fig. 6 *A*) and γ_5 (Fig. 6 *B*). In contrast, cyclooxygenase inhibitors did not significantly affect the carboxyl methylation of γ subunits in the presence of substimulatory concentrations [0.1 mM] of glucose (not shown). Furthermore, provision of exogenous PGE_2 (10 μM) completely reversed the inhibition by cyclooxygenase inhibitors of carboxyl methylation of γ subunits stimulated by glucose (Fig. 7 *A* and *B*). Under similar conditions, no additive effects of 11.1-mM glucose and PGE_2 on the carboxyl methylation of γ subunits were demonstrable (not shown), suggesting a common mode of action of these two modulators. In contrast, potassium-induced carboxyl methylation of γ_2 and γ_5 isoforms was resistant to inhibition of the cyclooxygenase pathway. The degree of carboxyl methylation of γ_2 and γ_5 isoforms in the presence of indomethacin plus K^+ (40 mM) was 89±5% and 98±10% ($n = 4$ determinations), respectively, of the control values observed in the presence of K^+ alone. Indomethacin only modestly inhibited (−18 to −26%; $n = 4$ determinations each) mastoparan-induced carboxyl methylation of γ_2 and γ_5 isoforms. Together, these above data indicate that glucose-stimulated carboxyl methylation of γ_2 and γ_5 isoforms may be due to dissociation of α/βγ subunits mediated by PGE_2, the synthesis of which is stimulated by glucose (49, 50), whereas calcium influx can (also) activate other pathways, presumably at least partially in a direct fashion as in the case of mastoparan (46).

Data above indicated that KCl (i.e., calcium)-induced stimulation of the carboxyl methylation of γ subunits was largely resistant to indomethacin, suggesting a mechanism different from the one involving PGE_2. Therefore, we examined whether it might be mediated by the lipid hydrolytic products of cal-

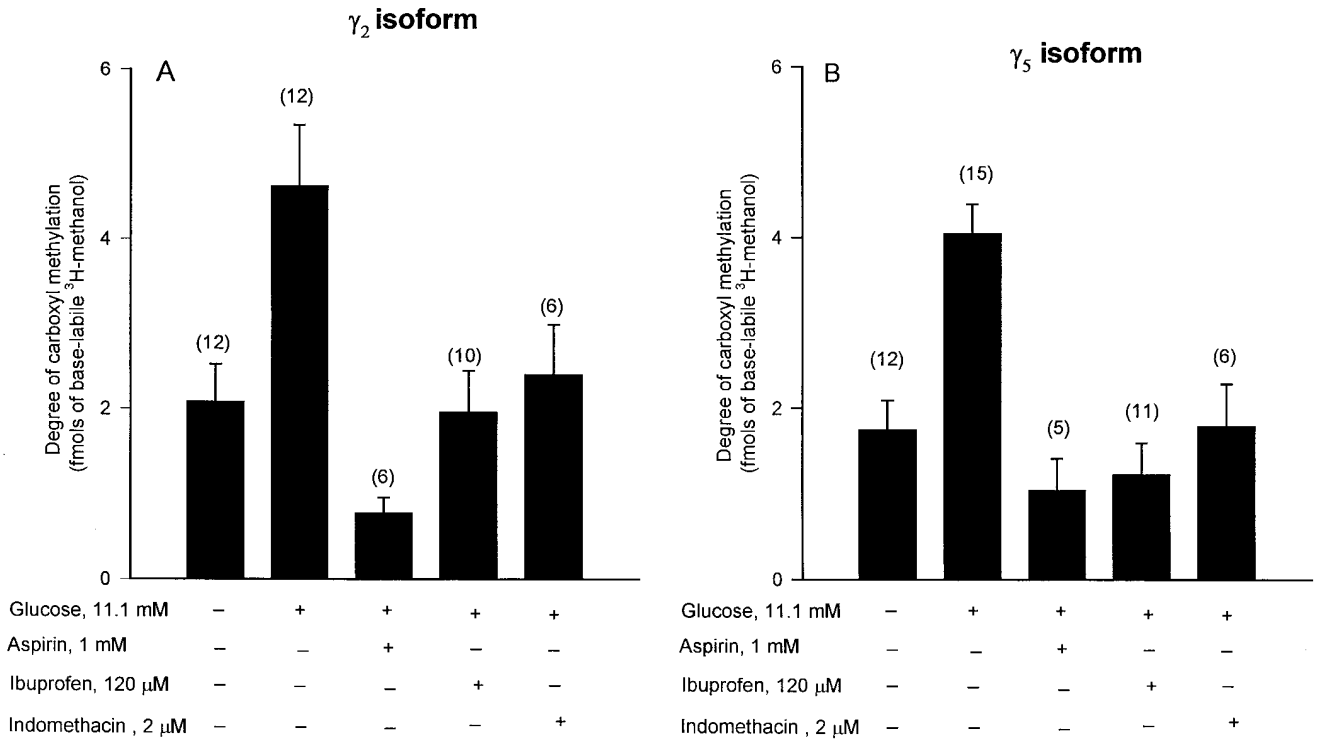

Figure 6. Inhibition by cyclooxygenase inhibitors of glucose-stimulated carboxyl methylation of γ subunit isoforms 2 (*A*) and 5 (*B*) in intact HIT cells. Intact HIT cells were prelabeled with [^{3}H]methionine for 60 min as indicated in the text. After prelabeling, they were incubated with glucose (0.1 mM or 11.1 mM) or glucose (11.1 mM) plus aspirin (1 mM), glucose (11.1 mM) plus ibuprofen (120 μM) or glucose (11.1 mM) plus indomethacin (2 μM) for 5 min as described in Methods. Reaction was terminated by the addition of extraction medium and γ_2 (*A*) and γ_5 (*B*) isoforms were immunoprecipitated (see Methods for details) and their carboxyl methylation was quantitated by base-labile methanol assay. Data are expressed as fmols of base-labile [^{3}H]methanol and are mean±SEM from individual immunoprecipitations in each case as indicated in the parentheses. Glucose significantly ($P < 0.001$) stimulated the carboxyl methylation of both γ_2 and γ_5 isoforms, an effect that was completely abolished by each of the three cyclooxygenase inhibitors tested.

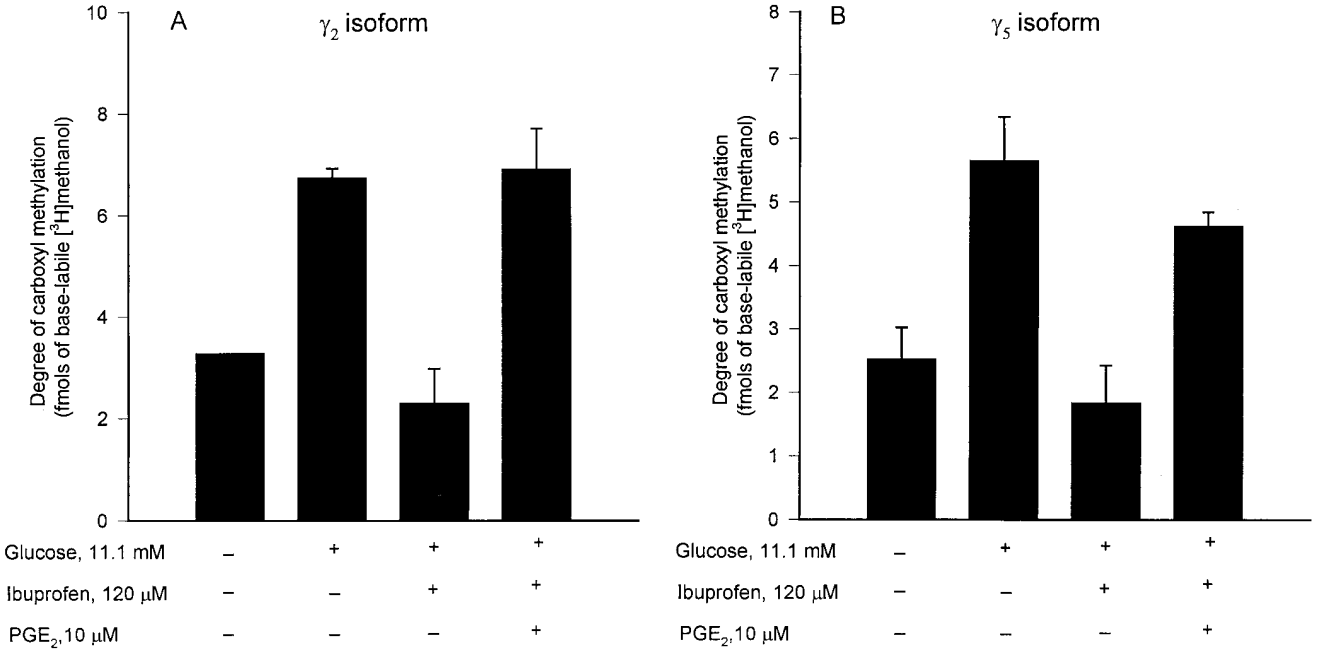

Figure 7. Reversal by exogenous PGE_2 of ibuprofen-induced inhibition of glucose-stimulated carboxyl methylation of γ_2 (*A*) and γ_5 (*B*) isoforms. Intact HIT cells were prelabeled with [^{3}H]methionine for 60 min as indicated in the text. After prelabeling, they were incubated with glucose, ibuprofen or PGE_2 in combinations as indicated in the figure (see Methods for additional details). Reaction was terminated by the addition of extraction medium and isoform 2 (*A*) and isoform (*B*) were immunoprecipitated and their degree of carboxyl methylation was quantitated by vapor phase equilibration assay. Data are mean±SEM of 4–8 individual immunoprecipitations from two independent experiments.

cium-sensitive phospholipases C (e.g., diacylglycerol [DAG]) or A_2 (e.g., lysophospholipids). For intact HIT cell studies, PMA (200 nM) was used in place of DAG; PMA had no significant effects on the carboxyl methylation of either γ_2 (128± 28% of control; $n = 3$ determinations) or γ_5 (98±2% of control; $n = 3$ determinations). Of all the lysophospholipids tested, only LPA significantly stimulated the carboxyl methylation of γ_2 subunit (Fig. 8). Additional studies indicated that the stimulatory effects of LPA were only modestly (but significantly) reduced in HIT cells exposed to PTx (254±10% in control cells versus 170±24% in PTx-treated cells; $P = 0.036$; df = 4). These data indicate yet another mechanism whereby the carboxyl methylation of γ subunits may be modulated in intact HIT cells, but do not unequivocally explain the underlying mechanism for a calcium-stimulated, PTx-sensitive carboxyl methylation of γ subunits. Indeed, similar PTx-insensitive mechanisms have been suggested for LPA signaling pathways in other cell types (51).

Discussion

In most cells, transduction of extracellular signals involves ligand binding to an exofascial receptor, often followed by activation of one or more G-proteins and their associated effector systems (1, 2). Pancreatic β cells are unusual in that the major physiologic agonist, glucose, lacks an extracellular receptor (52). Instead, events consequent to glucose metabolism promote insulin secretion via the generation of diffusible second messengers, such as purine nucleotides, cations, cyclic nucleotides, and lipid hydrolytic products generated by the action of phospholipases A_2, D, or C (53). Nonetheless, recent evidence indicates that glucose effects on insulin secretion from β cells involve G-proteins (21, 34, 35). However, no previous study has documented the activation by glucose or calcium (i.e., receptor-independent agonists) of trimeric G-protein(s).

There are at least three reports that described activation by glucose of protein carboxyl methylation in pancreatic β cells. Campillo and Ashcroft (54) first reported an increase in the carboxyl methylation of endogenous islet proteins by a combination of glucose (20 mM) and isobutyl methylxanthine (1 mM), but not by glucose alone. However, these investigators did not identify the proteins which were carboxyl methylated. More recent studies using normal rat islets, HIT cells (21, 35) as well as βTC3 cells (34) have reported glucose- and K^+-dependent, transient (peaking at < 1 min) stimulation of the carboxyl methylation of specific low molecular mass G-proteins. Immunoprecipitation studies have identified these proteins as Cdc42, Rac, and Rap. The carboxyl methylation of Rap was stimulated by glucose and K^+ (34, 35), whereas the carboxyl methylation of Cdc42 was stimulated only by glucose (35). We also observed that the carboxyl methylation of Rac was stimulated only by K^+ and not glucose (21). Our findings, that the carboxyl methylation of γ subunit was stimulated by both glucose and K^+, might indicate that a related mechanism of stimulation is involved as in the carboxyl methylation of Rap and/or Cdc42. The carboxyl methylation of γ subunits stimulated by glucose or K^+ required extracellular calcium, since its removal (using EGTA) attenuated this effect. Likewise, coprovision of calcium channel blockers (e.g., D-600) markedly reduced both glucose- and K^+-mediated increase in the carboxyl methylation of rap 1 in TC3 cells (34) and in HIT cells (Kowluru, A., and S.A. Metz, unpublished observations). It is possible that

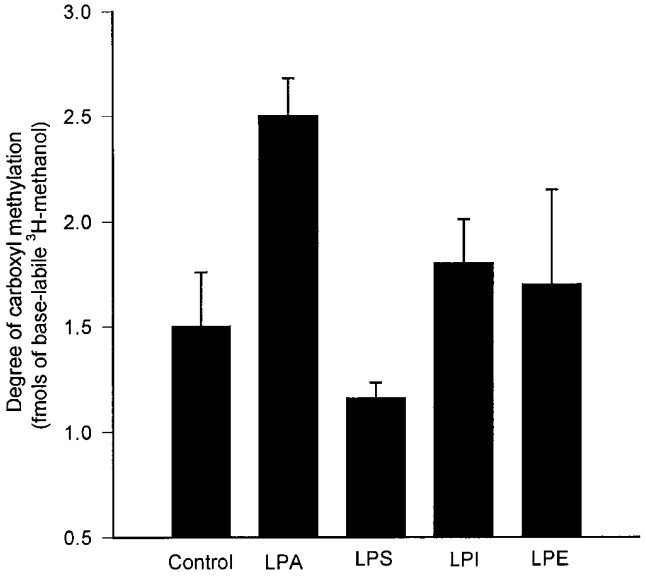

Figure 8. Selective stimulatory effects of LPA on the carboxyl methylation of γ_2 isoform in intact HIT cells. Intact HIT cells were prelabeled with [^{3}H]methionine for 60 min as indicated in the text. After prelabeling, they were incubated with 0.1-mM glucose alone (*Control*) or 0.1-mM glucose plus each of the lysophospholipids (10 μM; as indicated in the figure) for 30 min at 37°C. Reaction was terminated by the addition of extraction medium and γ_2 isoform was immunoprecipitated and the degree of carboxyl methylation was quantitated by base-labile methanol assay. Data are mean±SEM of three individual immunoprecipitations in each case. Of all the lysophospholipids tested, only LPA significantly stimulated the carboxyl methylation of γ_2 subunit ($P < 0.001$). *LPA*, lysophosphatidic acid; *LPS*, lysophosphatidylserine; *LPI*, lysophosphatidylinositol; *LPE*, lysophosphatidylethanolamine.

the carboxyl methylation of these two proteins (i.e., Rap and γ subunits) is interdependent in the cascade of events leading to calcium-mediated insulin secretion. Indeed, recent studies (55, 56) have proposed possible cross-talk between low molecular mass and trimeric G-proteins in cell activation. Interestingly, however, in these studies the input via low molecular mass G-proteins was usually distal to effects on trimeric G-proteins (possibly via βγ complex) whereas in our current study, Cdc42 and/or Rap seem(s) to be acting proximal to G_i/G_o (Fig. 9). Since calcium removal vitiated effects of glucose or K^+ but not mastoparan, and PTx attenuated effects of all the three agonists, one can infer that calcium entry can activate (i.e., promote the carboxyl methylation of) a mastoparan- and PTx-sensitive trimeric G-protein, probably belonging to the subclass of G_i or G_o (46), albeit by dissimilar mechanisms (i.e., PGE_2-dependent mechanism for glucose; largely PGE_2-independent mechanism for potassium and mastoparan).

These data are in agreement with recent observations of Philips et al. (57) who reported stimulation of the carboxyl methylation of $G\gamma_2$ isoform in neutrophils by GTPγS (which was potentiated further by FMLP). In this study, we also observed stimulatory effects of GTPγS or PGE_2 on the carboxyl methylation of γ subunits using cell-free preparations and intact islets, but they were not additive, suggesting that GTP might work via the generation of PGE_2. Furthermore, using MPA, an inhibitor of GTP biosynthesis, in intact cells, we observed that a selective depletion of GTP markedly reduced the stimulatory effects of either glucose or K^+, but not of PGE_2 itself, on the carboxyl methylation of γ subunits. Coprovision of guanosine (which restores intracellular GTP levels and GTP/GDP ratio) completely restored the ability of glucose to stimulate the carboxyl methylation of γ subunits. Interestingly, inhibitory effects of MPA were demonstrable within 3 h of exposure. Under these conditions, we observed a marked reduction in GTP levels and GTP/GDP ratios (Li, G., and S.A. Metz, unpublished observations). These data clearly imply a regulatory role for intracellular GTP in glucose-mediated regulation of γ subunit carboxyl methylation in intact cells. Since we observed that PGE_2-mediated stimulation of the carboxyl methylation of γ subunits was resistant to MPA (see Results), it seems likely that GTP is required at a step proximal to PGE_2 synthesis. Thus we surmise (Fig. 9) that glucose, probably by increasing GTP/GDP ratio (58), can promote PGE_2 synthesis as documented by Wolf et al. (59). Presumably, adequate GTP is required to activate a phospholipase which releases AA as substrate for PGE_2 synthesis. Indeed, glucose increases AA release (50, 51), via activation of PLC (60) and/or PLA_2. We have demonstrated that GTP depletion inhibits the activation of PLC, probably by blockade of the carboxyl methylation of Cdc42 and/or Rap (35). Furthermore, it is possible that, by increasing the GTP/GDP ratio (58), glucose favors the formation of GTP-bound (active) conformation of G-proteins in contrast to their GDP-bound (inactive) conformation. Recently, Bond et al. (61) proposed such model systems for G-protein coupled receptor activation involving back-and-forth transition of the inactive receptor conformation to a spontaneously active conformation of the receptor.

Our data also indicate that in addition to GTP, extracellular calcium may be involved in the regulation of carboxyl methylation of γ subunits by glucose and potassium. We tentatively propose that this may represent the requirement for calcium entry for phospholipase activation in β cells. Interestingly, however, addition of calcium (up to 100 μM) did not stimulate the carboxyl methylation of γ subunits in cell-free preparations (additional data not shown). These data may imply that additional intermediary factor(s) are needed for calcium-mediated regulation of phospholipases and/or the carboxyl methylation of γ subunits, which are vitiated after homogenization/sonication. Additional studies are needed to address these differences between broken cell preparations and intact cells with regard to their differential responsiveness to calcium. However, as in studies of neutrophils (57), our data indicate that important events in β cells could easily be overlooked not only if intact cells were not studied, but also if the carboxyl methylation of specific γ subunit isoforms had not been quantified. While the exact mechanism whereby mastoparan stimulates the carboxyl methylation of γ subunits is unknown, recent studies by Eddlestone et al. (62) have suggested that mastoparan, among other actions, increases intracellular calcium in intact HIT and RINm5F cells by inhibiting ATP-sensitive K^+ channels. However, unlike glucose or potassium, mastoparan-stimulated carboxyl methylation of γ subunits does not seem to require extracellular calcium, implying a different mechanism which presumably involves a direct activation of G_i or G_o (46).

Additional findings indicated that PTx pretreatment of in-

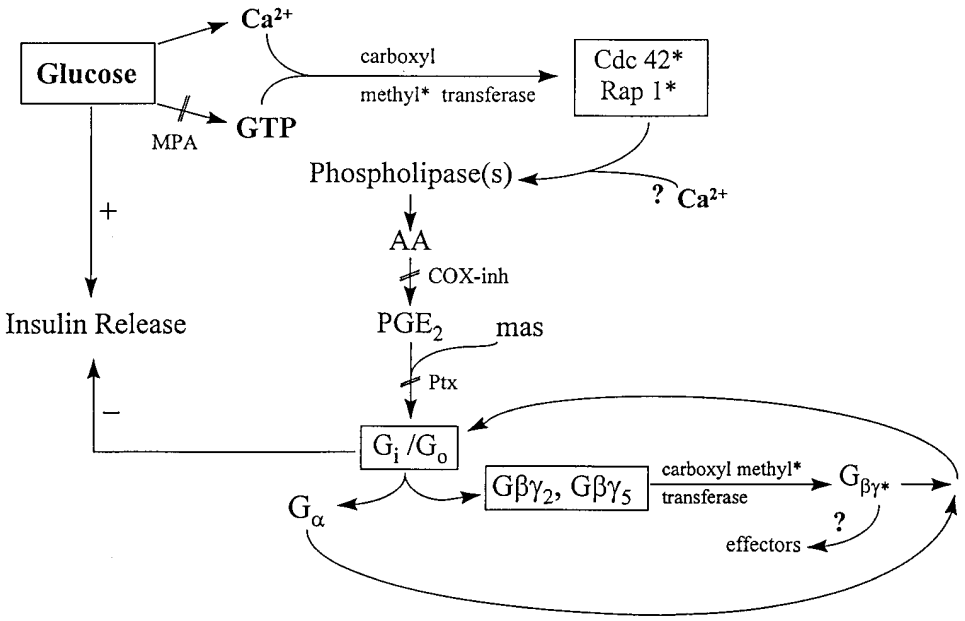

Figure 9. Schema depicting postulated sequence of events whereby glucose activates the carboxyl methylation of γ subunits, and its possible relevance to insulin secretion. Glucose stimulates the carboxyl methylation of the low molecular mass G-proteins, Cdc42 (35) and rap (34, 35), an effect abrogated by depletion of intracellular GTP stores (35). D600, a voltage-dependent calcium channel blocker, abolishes glucose's stimulation of the carboxyl methylation of Cdc42 (Kowluru, A., and S.A. Metz, unpublished observations), suggesting a regulatory role for calcium fluxes in the carboxyl methylation of these proteins. Presumably, calcium stimulates GTP binding to Cdc42, possibly via a GTP-exchange factor leading to translocation of Cdc42 from cytosol to membrane site of methyl transferase and increasing its affinity for that enzyme (36). This effect is insensitive to PTx (Kowluru, A., and S.A. Metz, unpublished observations). The carboxyl methylation of specific proteins (e.g., Cdc42) results in augmented phospholipase activity (35). This effect would augment release of AA from membrane phospholipids (50, 51); this is a second possible stimulatory site of action of calcium. AA is the substrate for the biosynthesis of PGE_2 via the cyclooxygenase pathway (sensitive to COX-inh). The PGE_2 thus generated activates PTx-sensitive, but MPA-insensitive (79), trimeric G-proteins in HIT cells (e.g., G_i and G_o) resulting in the dissociation of α subunits from the free βγ complex. These same G-proteins are directly activated by mastoparan (*mas*) but in a PGE_2-independent fashion. Free α subunits of such inhibitory G-proteins (e.g., G_i/G_o) inhibit glucose-induced insulin release (24, 25). βγ, as a dimer, is the preferred substrate for carboxyl methylation (this study). The complexity of glucose's effects on insulin secretion do not permit their inclusion in the figure. The carboxyl methylated (and hence more hydrophobic) γ subunits reassociate with α subunits to regain the trimeric conformation in preparation for a new cycle of stimulation. Methylation of βγ may also increase its interaction with putative effector systems (33). *Methyl group (transferred to prenylcysteine moiety at the C terminus).

tact cells reduced the ability of glucose, K^+, or mastoparan to stimulate the carboxyl methylation of γ subunits. These data are in agreement with observations of Phillips et al. (63) who reported abolition of GTPγS-stimulated carboxyl methylation of γ subunits in neutrophils pretreated with PTx. Several recent studies have immunologically localized PTx-sensitive α subunits of trimeric G-proteins in normal rat islets, human islets, and β clonal cells (19, 23, 25). Together, the positive effects of mastoparan or GTPγS on the carboxyl methylation and the negative effects of PTx clearly indicate that free βγ dimer is the preferred substrate for γ subunit to be carboxyl methylated.

Data obtained using secretory granule fraction provide evidence for the carboxyl methylation of a 5–7-kD protein, presumably representing the γ subunits. These observations are compatible with recent studies on immunological identification of both α and β subunits of trimeric G-proteins in the secretory granule fraction of normal rat islets and insulinoma tissue (22), and βTC3 cells (48), and provide further evidence for not only the presence of all three subunits of αβγ trimer on secretory granules, but also their (functional) regulation by carboxyl methylation (this study) and phosphorylation (32). Immunoblotting data indicated that at least four isoforms of γ subunits (i.e., γ_1, γ_2, γ_5, and γ_7) are localized in pancreatic β cells (Fig. 1). These data are compatible with recent reports of the distribution of γ_2, γ_5, and γ_7 isoforms in several tissues, including the pancreas (64). Interestingly, γ_1 isoform was previously thought to be exclusively localized in the retina. However, Zigman et al. (65) have demonstrated the expression of α subunits of retinal trimeric G-proteins (e.g., G_t) in normal rat islets. Thus, it seems likely that (subunits of) transducin-like G-proteins may be localized in nonretinal tissues, including the pancreatic β cell. We failed to detect the γ_3 isoform in β cells; this is compatible with several recent reports suggesting an exclusive localization of this isoform in the brain (64). Our findings that the carboxyl methylation of each of these isoforms was differentially modulated (i.e., stimulated or inhibited) by glucose, potassium, or mastoparan are particularly important. The significance of the observed decrease in the carboxyl methylation of γ_7 isoform induced by glucose or glucose in the PTx-pretreated HIT cells is not clear at this time, but may be due to glucose-induced release of AA. In contrast to the stimulatory effects of glucose on carboxyl methylation, unmodified AA (but not its methyl ester or other fatty acids) induces a concentration-dependent inhibition of γ subunit carboxyl methylation (Kowluru, A., and S.A. Metz, unpublished observations). Interestingly, we observed previously that AA also inhibits phospholipid methyl transferase activity in normal rat islets (66), a finding confirmed recently by Choi and Gibbons (67) using polymorphonuclear leukocyte membranes. Further studies will be needed to elucidate the physiologic relevance (if any) of the inhibitory effects of AA on methyl transferase activity. Clearly they cannot be attributed to generation of prostaglandins.

We also describe herein the presence of a demethylating system in normal rat islet homogenates. The half-life (3.5–4.0 h) of the methylated γ subunit is relatively long (at least in the basal state) compared to that of low molecular mass G-proteins (e.g., a $t_{1/2}$ of < 2 h) or the catalytic subunit of protein phosphatase 2A (40 min; see reference 40). However, it is possible that one (or more) individual isoforms is demethylated more rapidly. Using purified βγ subunits, Parish and coworkers (68, 69) have proposed that the demethylating enzyme in rod-outer segments may be similar to pig liver esterase. These investigators demonstrated that pig liver esterase can hydrolyze the methyl ester bond at the C terminus of γ subunit more efficiently when βγ complex is dissociated from the α subunit, suggesting masking of the methylated C-terminus region during the complexation of αβγ subunits. Such putative steric hindrance may be one of the contributing reasons for the slower rates of demethylation of γ subunits in comparison to relatively rapid demethylation of monomeric G-proteins (68, 69).

In summary, this study identifies, for the first time, the presence of a methylation-demethylation cycle for selected γ subunits of trimeric G-proteins in rodent and human pancreatic β cells that is initiated by nonreceptor agonists (e.g., glucose, mastoparan, and calcium). Such regulatory effects seem to require extracellular calcium and intracellular GTP, presumably to activate AA-releasing phospholipases. Additionally, PTx pretreatment markedly attenuated the stimulatory effects by these agents, suggesting that these agonists regulate carboxyl methylation of γ subunits that belong to the inhibitory class of trimeric G-proteins (especially G_i or G_o). Thus, glucose, in addition to dominant positive signals for insulin release, also provides endogenous negative modulators (e.g., PGE_2). Indeed, in β cells, inhibition of PGE_2 synthesis potentiates glucose-induced insulin release (50, 51, 70, 71).

The functional consequences of γ subunit methylation are uncertain and may be complex. The posttranslational modification of γ subunits has been implicated in the interaction of the βγ dimers with their α subunits (72, 73), their membrane association (74), and the coupling to receptors or effectors, including PLC (75), PI kinase (9), and βARK (76). Thus, the carboxyl methylation of γ could play roles not only in transducing physiologic effects of αβγ subunits, but also in signal termination and/or desensitization. The biological significance of carboxyl methylation of the γ subunit is not addressed by our study. It is possible that as with low molecular mass G-proteins (35), an increase in carboxyl methylation of (β)γ could increase interaction with its putative effectors. We do not favor this formulation, however, for two reasons: (*a*) recent studies suggest that the carboxyl methylation of (β)γ results in only modest increase in effector activity (77), and (*b*) inhibition of the carboxyl methylation of γ subunits using AFC or by MPA in islets did not block action of the G_i or G_o agonist (e.g., epinephrine) or G_q agonist (e.g., carbachol) on insulin release (20). Rather, we speculate that, by promoting membrane interaction and reassociation of βγ with α subunits, an increase in the carboxyl methylation might provide an off signal terminating the activation of trimeric G-proteins and thereby facilitating reassembly of the αβγ trimer to prepare the cell for a new cycle of G-protein activation. Thus, as indicated by the findings of Lederer et al. (78) blockade of carboxyl methylation of G_γ subunits is reduced FMLP-stimulated GTP binding and hydrolysis, and PTx-stimulated ADP ribosylation. Further studies will be needed to unravel these complexities.

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Reference

1. Gilman, A.G. 1987. G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* 56:615–649.

2. Birnbaumer, L. 1992. Receptor-to-effector signaling through G proteins: roles for βγ dimers as well as α subunits. *Cell.* 71:1069–1072.

3. Ikeda, S.R. 1996. Voltage-dependent modulation of N-type calcium channels by G-protein βγ subunits. *Nature (Lond.).* 380:255–258.

4. Weiland, T., M. Rozani, and K.H. Jakobs. 1992. Stimulation and inhibition of platelet adenylyl cyclase by thiophosphorylated transducin βγ subunits. *J. Biol. Chem.* 267:20791–20797.

5. Clapham, D.E., and E.J. Neer. 1993. New roles for G-protein βγ-dimers in transmembrane signaling. *Nature (Lond.).* 365:403–406.

6. Blank, J.L., K.A. Brattain, and J.H. Exton. 1992. Activation of cytosolic phosphoinositide phospholipase C by G-protein βγ subunits. *J. Biol. Chem.* 267:23069–23075.

7. Olson, S.C., S.R. Tyagi, and J.D. Lambeth. 1990. Fluoride activates diacylglycerol and superoxide genearation in human neutrophils via PLD/PA phosphohydrolase-dependent and -independent pathways. *FEBS Lett.* 272:19–24.

8. Ito, A., T. Satoh, Y. Kaziro, and H. Itoh. 1995. G protein βγ subunit activates Ras, Raf, and MAP kinase in HEK 293 cells. *FEBS Lett.* 368:183–187.

9. Stephens, L., A. Smrcka, F.T. Cooks, T.R. Jackson, P.C. Sternweis, and P.C. Hawkins. 1994. A novel phosphoinositide 3 kinase activity in myeloid-derived cells is activated by G protein βγ subunits. *Cell.* 77:83–93.

10. Manser, E., T. Leung, H. Salihuddin, L. Tan, and L. Lim. 1993. A nonreceptor tyrosine kinase that inhibits the GTPases activity of $p21^{cdc42}$. *Nature (Lond.).* 363:364–367.

11. Zhang, S., J.M. Han, M.A. Sells, J. Chernoff, U.G. Knaus, R.J. Uhevitch, and G.M. Bokoch. 1995. Rho family GTPases regulate p38 MAP kinase through the downstream mediator Pak 1. *J. Biol. Chem.* 270:23934–23936.

12. Amano , M., H. Mukai, Y. Ono, K. Chihara, T. Matsui, Y. Hamajima, K. Okawa, A. Iwamatsu, and K. Kaibuchi. 1996. Identification of a putative target for Rho as the serine-threonine kinase protein kinase N. *Science (Wash. DC).* 271:648–650.

13. Malcolm, K.C., C.M. Elliott, and J.H. Exton. 1996. Evidence for Rho-mediated agonist stimulation of phospholipase D in Rat1 fibroblasts. *J. Biol. Chem.* 271:13135–13139.

14. Tolias, K.F., L.C. Cantley, and C.L. Carpenter. 1995. Rho family GTPases bind to phosphoinositide kinases. *J. Biol. Chem.* 270:17656–17659.

15. Kowluru, A., and S.A. Metz. 1994. Stimulation by prostaglandin E2 of a high-affinity GTPase in the secretory granules of normal rat and human pancreatic islets. *Biochem. J.* 297:399–406.

16. Kowluru, A., and S.A. Metz. 1994. Characterization of nucleoside diphosphokinase activity in human and rodent pancreatic β cells: evidence for its role in the formation of guanosine triphosphate, a permissive factor for nutrient-induced insulin secretion. *Biochemistry.* 33:12495–12503.

17. Kowluru, A., M.E. Rabaglia, K. Muse, and S.A. Metz. 1994. Subcellular localization and kinetic characterization of guanine nucleotide binding proteins in normal rat and human pancreatic islets and transformed beta cells. *Biochim. Biophys. Acta.* 1222:348–359.

18. Kowluru, A., and S.A. Metz. 1994. Regulation of guanine-nucleotide binding proteins in islet subcellular fractions by phospholipase-derived lipid mediators of insulin secretion. *Biochim. Biophys. Acta.* 1222:360–368.

19. Kowluru, A., and S.A. Metz. 1994. GTP and its binding proteins in the regulation of insulin exocytosis. *In* Molecular Biology of Diabetes. B. Draznin and D. LeRoith, editors. The Humana Press, Inc., Totowa, New Jersey. 249–283.

20. Metz, S.A., M.E. Rabaglia, J.B. Stock, and A. Kowluru. 1993. Modulation of insulin secretion from normal rat islets by inhibitors of post-translational modifications of GTP-binding proteins. *Biochem. J.* 295:31–40.

21. Kowluru, A., G. Li, M. Rabaglia, V.B. Segu, F. Hofmann, K. Aktories, and S.A. Metz. 1997. Evidence for differential roles of the Rho subfamily of GTP-binding proteins in glucose- and calcium-induced secretion from pancreatic β cells. *Biochem. Pharmacol.* In press.

22. Kowluru, A., S.E. Seavey., C.J. Rhodes., and S.A. Metz. 1996. A novel regulatory mechanism for trimeric GTP-binding proteins in the membrane and

secretory granule fractions of human and rodent β cell. *Biochem. J.* 313:97–107.

23. Seaquist, E.R., T.F. Walseth, B. Redmon, and R.P. Robertson. 1994. G protein regulation of insulin secretion. *J. Lab. Clin. Med.* 123:338–345.

24. Robertson, R.P., E.R. Seaquist, and T.F. Walseth. 1991. G proteins and modulation of insulin secretion. *Diabetes.* 40:1–6.

25. Robertson, R.P., E.R. Seaquist, T. Walseth, H.-J. Zhang, B.J. Redmon, V. Poitoit, and D.M. Kendall. 1996. GTP-binding proteins and regulation of pancreatic islet function. Heterotrimeric proteins, insulin gene expression, and insulin secretion. *In* Diabetes Mellitus. D. Leroith, S.I. Taylor, and J.M. Olefsky, editors. Lippincott-Raven Publishers, Philadelphia. 49–56.

26. Takai, Y., K. Kaibuchi, A. Kikuchi, and M. Kawata. 1992. Small GTP-binding proteins. *Int. Rev. Cytol.* 133:187–230.

27. Wedengaertner, P.B., P.T. Wilson, and H.R. Bourne. 1995. Lipid modifications of trimeric G proteins. *J. Biol. Chem.* 270:503–506.

28. Kowluru, A., R. Kowluru, and A. Yamazaki. 1992. Functional alterations of G-proteins in diabetic rat retina: a possible explanation for the early visual abnormalities in diabetes mellitus. *Diabetologia.* 35:624–631.

29. Fields, T.A., and P.J. Casey. 1995. Phosphorylation of Gzα by protein kinase C blocks interaction with the βγ complex. *J. Biol. Chem.* 270:23119–23125.

30. Weiland, T., B. Nurnberg, I. Ulibarri, S. Kaldenberg-Stasch, G. Schultz, and K.H. Jakobs. 1993. Guanine nucleotide-specific phosphate transfer by guanine nucleotide-binding regulatory protein β subunits. Characterization of the phosphorylated amino acid. *J. Biol. Chem.* 268:18111–18118.

31. Hohenegger, M., T. Mitterauer, T. Voss, C. Nanoff, and M. Freissmuth. 1996. Thiophosphorylation of the G protein β subunit in human platelet membranes: evidence against a direct phosphate transfer reaction to Gα subunits. *Mol. Pharmacol.* 49:73–80.

32. Morishita, R., H. Nakayama, T. Isobe, T. Matsuda, Y. Hashimoto, T. Okano, Y. Fukada, K. Mizuno, S. Ohno, O. Kozawa, et al. 1995. Primary structure of a γ subunit of G protein, γ12, and its phosphorylation by protein kinase C. *J. Biol. Chem.* 270:29469–29475.

33. Rando, R.R. 1996. Chemical biology of protein isoprenylation/methylation. *Biochim. Biophys. Acta.* 1300:5–16.

34. Leiser, M., S. Efrat, and N. Fleischer. 1995. Evidence that Rap1 carboxylmethylation is involved in regulated insulin secretion. *Endocrinology.* 136: 2521–2530.

35. Kowluru, A., S.E. Seavey, G. Li, R.L. Sorenson, A.J. Weinhaus, R. Nesher, M.E. Rabaglia, J. Vadakekalam, and S.A. Metz. 1996. Glucose- and GTP-dependent stimulation of the carboxyl methylation of CDC42 in rodent and human pancreatic islets and pure β cells. Evidence for an essential role of GTP-binding proteins in nutrient-induced insulin secretion. *J. Clin. Invest.* 98: 540–555.

36. Li, G., A. Kowluru, and S.A. Metz. 1996. Characterization of prenylcysteine methyltransferase in insulin-secreting cells. *Biochem. J.* 316:345–351.

37. Metz, S.A., M.E. Rabaglia, and T.J. Pintar. 1992. Selective inhibitors of GTP synthesis impede exocytotic insulin release from intact rat islets. *J. Biol. Chem.* 267:12517–12527.

38. Gillison, S.L., and G.W.G. Sharp. 1994. ADP ribosylation by cholera toxin identifies three G-proteins that are activated by galanin receptor. *Diabetes.* 43:24–32.

39. Robishaw, J.D., and E.A. Balcueva. 1994. Preparation, characterization, and use of antibodies with specificity for G-protein γ subunits. *Methods Enzymol.* 237:498–509.

40. Kowluru, A., S.E. Seavey, M.E. Rabaglia, R. Nesher, and S.A. Metz. 1996. Carboxyl methylation of the catalytic subunit of protein phosphatase 2A in insulin-secreting cells: evidence for functional consequences on enzyme activity and insulin secretion. *Endocrinology.* 137:2315–2323.

41. Li, G.D., H. Hidaka, and C.B. Wollheim. 1992. Inhibition of voltage-gated Ca^{2+} channels and insulin secretion in HIT cells by the Ca^{2+}/calmodulin–dependent protein kinase II inhibitor KN-62: comparison with antagonists of calmodulin and L-type Ca^{2+} channels. *Mol. Pharmacol.* 42:489–498.

42. Grynkiewicz, G., M. Ponie, and R.Y. Tsien. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440–3450.

43. Bradford, M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

44. Ray, K., C. Kunsch, L.M. Bonner, and J.D. Robishaw. 1995. Isolation of cDNA clones encoding eight different human G protein γ subunits, including three novel forms disignated the γ4, γ10, and γ11 subunits. *J. Biol. Chem.* 270: 21765–21777.

45. Tan, E.W., and R.R. Rando. 1992. Identification of an isoprenylated cysteine methyl ester hydrolase activity in bovine rod outer segment membranes. *Biochemistry.* 31:5572–5578.

46. Hagashijima, T., J. Burnier, and E.M. Ross. 1990. Regulation of Gi and Go by masoparan, related amphiphilic peptides and hydrophobic amines. *J. Biol. Chem.* 265:14176–14186.

47. Komatsu, M., A.M. McDermott, S.L. Gillison, and G.W.G. Sharp. 1993. Mastoparan stimulates exocytosis at a Ca^{2+}-independent late site in stimulus-secretion coupling. *J. Biol. Chem.* 268:23297–23306.

48. Konrad, R.J., R.A. Young, R.D. Record, R.M. Smith, P. Butkerai, D. Manning, L. Jarret, and B.A. Wolf. 1995. The heterotrimeric G protein Gi is localized to the insulin secretory granules of β cells and is involved in exocytosis. *J. Biol. Chem.* 270:12869–12876.

49. Metz, S.A., R.P. Robertson, and W.Y. Fujimoto. 1981. Inhibition of prostaglandin E synthesis augments glucose-induced insulin secretion in cultured pancreas. *Diabetes.* 30:551–555.

50. Turk, J., J.A. Hughes, R.A. Easom, B.A. Wolf, D.W. Scharp, P.E. Lacy, and M.L. McDaniel. 1988. Arachidonic acid metabolism and insulin secretion by isolated human pancreatic islets. *Diabetes.* 37:992–996.

51. Kumagai, N., N. Morii, T. Ishizaki, N. Watanabe, K. Fujisawa, Y. Saito, and S. Narumiya. 1995. Lysophosphatidic acid-induced activation of protein Ser/Thr kinases in cultured rat 3Y1 fibroblasts. Possible involvement in rho p21-mediated signaling. *FEBS Lett.* 366:11–16.

52. Hedescov, C.J. 1980. Mechanism of glucose-induced insulin secretion. *Physiol. Rev.* 60:442–509.

53. Prentki, M., and F.M. Matschinsky. 1987. Ca^{2+}, cAMP, and phospholipid derived messengers in coupling mechanisms of insulin secretion. *Physiol. Rev.* 67:1185–1248.

54. Campillo, J.E., and S.J.H. Ashcroft. 1982. Protein carboxyl methylation in rat islets of Langerhans. *FEBS Lett.* 134:71–75.

55. Colombo, M.I., J. Inglese, C. D'Souza-Schores, W. Beron, and P.D. Stahl. 1995. Heterotrimeric G proteins interact with the small GTPase ARF. Possibilities for the regulation of vesicular traffic. *J. Biol. Chem.* 270:24504–24571.

56. Bokoch, G.M. 1996. Interplay between Ras-related and heterotrimeric GTP-binding proteins: lifestyles of the BIG and little. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 10:1290–1295.

57. Philips, M.R., R. Staud, M. Pillinger, A. Feoktistov, C. Volker, J. Stock, and G. Weissmann. 1995. Activation-dependent carboxyl methylation of neutrophil G-protein γ subunit. *Proc. Natl. Acad. Sci. USA.* 92:2283–2287.

58. Detimary, P., V.D. Berghe, and J.C. Henquin. 1996. Concentration dependence and time course of the effects of glucose on adenine and guanine nucleotides in mouse pancreatic islets. *J. Biol. Chem.* 271:20559–20565.

59. Wolf, B.A., S.M. Pasquale, and J. Turk. 1991. Free fatty acid accumulation in secretagogue-stimulated pancreatic islets and effects of arachidonate on depolarization-induced insulin secretion. *Biochemistry.* 30:6372–6379.

60. Vadakekalam, J., M.E. Rabaglia, Q.-H. Chen, and S.A. Metz. 1996. Glucose-induced phosphoinositide hydrolysis. A requirement for GTP in calcium-induced phospholipase activation in pancreatic islets. *Am. J. Physiol.* 271:E85–E95.

61. Bond, R.A., P. Leff, T.D. Johnson, C.A. Milano, H.A. Rockman, T.R. McMinn, S. Apparsundaram, M.F. Hyek, T.P. Kenakin, L.F. Allen, and R.J. Lefkowitz. 1996. Physiologic effects of inverse agonists in transgenic mice with myocardial overexpression of the β2-adrenoreceptor. *Nature (Lond.).* 374:272–276.

62. Eddlestone, G.T., M. Komatsu, L. Shen, and G.W.G. Sharp. 1995. Mastoparan increases the intracellular free calcium concentration in two insulin-secreting cell lines by inhibition of ATP-sensitive potassium channels. *Mol. Pharmacol.* 47:787–797.

63. Philips, M.R., R. Staud, M.H. Pillinger, and G. Weissmann. 1992. Prenylation and carboxyl methylation of a putative G_γ in human neutrophil membranes. *Clin. Res.* 40:250A.

64. Morishita, R., Y. Fukada, K. Kokame, T. Yoshizawa, K. Masuda, M. Niwa, K. Kato, and T. Asano. 1992. Identification and isolation of common and tissue-specific geranylgeranylated γ subunits of guanine-nucleotide-binding regulatory proteins in various tissues. *Eur. J. Biochem.* 210:1061–1069.

65. Zigman, J.M., G.T. Westermark, J. LaMendola, and D.F. Steiner. 1994. Expression of cone transducin, Gzα and other G-protein α subunit messenger ribonucleic acids in pancreatic islets. *Endocrinology.* 135:31–37.

66. Kowluru, A., R.S. Rana, and M.J. MacDonald. 1985. Phospholipid methyl transferase activity in pancreatic islets: activation by calcium. *Arch. Biochem. Biophys.* 24:72–81.

67. Choi, G., and W.A. Gibbons. 1991. Arachidonic acid as a feedback inhibitor of phospholipid methylation in rat polymorphonuclear leukocytes. *Biochem. Soc. Trans.* 19:308S.

68. Parish, C.A., and R.R. Rando. 1994. Functional significance of G protein carboxyl methylation. *Biochemistry.* 33:9986–9991.

69. Parish C.A., A.V. Smrcka, and R.R. Rando. 1995. Functional significance of βγ-sununit carboxyl methylation for the activation of phospholipase C and phosphoinositide 3- kinase. *Biochemistry.* 34:7722–7727.

70. Robertson, R.P., and M. Chen. 1977. A role for prostaglandin E in defective insulin secretion and carbohydrate intolerance in diabetes mellitus *J. Clin. Invest.* 60:743–753.

71. Robertson, R.P., D.J. Gavareski, D. Porte, and E.L. Bierman. 1974. Inhibition of in vivo insulin secretion by prostaglandin E1. *J. Clin. Invest.* 54:310–315.

72. Fukada, Y., T. Matsuda, K. Kokame, T. Takao, Y. Shimonishi, T. Akino, and T. Yoshizawa. 1994. Effects of carboxyl methylation of photoreceptor G protein γ subunit in visual transduction. *J. Biol. Chem.* 269:5163–5170.

73. Kisselev, O., M. Ermolaeva, and N. Gautam. 1995. Efficient interaction with a receptor requires a specific type of prenyl group on the G protein γ subunit. *J. Biol. Chem.* 270:25356–25358.

74. Muntz, K.H., P.C. Sternweis, A.G. Gilman, and S.M. Mumby. 1992. Influence of gamma subunit prenylation on association of guanine nucleotide-binding regulatory proteins with membranes. *Mol. Biol. Cell.* 3:49–61.

75. Smrcka, A.V., and P.C. Sternweis. 1993. Regulation of purified subtypes of phosphatidylinositol-specific phospholipase C beta by G protein αβγ subunits *J. Biol. Chem.* 268:9667–9674.

76. Lefkowitz, R.J. 1993. G protein coupled receptor kinases. *Cell.* 74:409–412.

77. Parish, C.A., A.V. Smrcka, and R.R. Rando. 1996. The role of G protein methylation in the function of geranylgeranylated βγ isoform. *Biochemistry.* 35: 7499–7505.

78. Lederer, E.D., A. Jacobs, J.L. Hoffman, G.B. Harding, J.D. Robishaw, and K.R. McLeish. 1994. Role of carboxyl methylation in chemoattractant receptor-stimulated G protein activation and functional response. *Biochem. Biophys. Res. Commun.* 200:1604–1614.

79. Metz, S.A., and A. Kowluru. 1996. GTP-binding proteins in the regulation of pancreatic β cell function. Putative roles for low molecular weight proteins. *In* Diabetes Mellitus. D. LeRoith, S.I. Taylor, and J.M. Olefsky, editors. Lippincott-Raven Publishers. Philadelphia. 57–68.