Gαo represses insulin secretion by reducing vesicular docking in pancreatic β cells

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**Running title:** Gαo regulates insulin vesicle docking

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Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org

Submitted 23 November 2009 and accepted 1 July 2010.

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Objective: Pertussis toxin (PTX) uncoupling-based studies have shown that G\(\alpha_i\) and G\(\alpha_o\) can inhibit insulin secretion in pancreatic \(\beta\) cells. Yet it is unclear whether G\(\alpha_i\) and G\(\alpha_o\) operate through identical mechanisms and how these G protein-mediated signals inhibit insulin secretion \textit{in vivo}. Our objective is to examine whether/how G\(\alpha_o\) regulates islet development and insulin secretion in \(\beta\) cells.

Research Design and Methods: Immunoassays were utilized to analyze the G\(\alpha_o\) expression in mouse pancreatic cells. G\(\alpha_o\) was specifically inactivated in pancreatic progenitor cells by pancreatic cell specific gene deletion. Hormone expression and insulin secretion in response to various stimuli were assayed \textit{in vivo} and \textit{in vitro}. Electron microscope and total internal reflection fluorescence-based assays were utilized to evaluate how G\(\alpha_o\) regulates insulin vesicle docking and secretion in response to glucose stimulation.

Results: Islet cells differentiate properly in G\(\alpha_o^{-/-}\) mutant mice. G\(\alpha_o\) inactivation significantly enhances insulin secretion both \textit{in vivo} and in isolation. G\(\alpha_o\) nullizygous \(\beta\) cells contain an increased number of insulin granules docked on the cell plasma membrane, although the total number of vesicles per \(\beta\) cell remains unchanged.

Conclusion: G\(\alpha_o\) is not required for endocrine islet cell differentiation but it regulates the number of insulin vesicles docked on \(\beta\) cell membrane.

Nutritional signals, including glucose and amino acids, are the major inducers for insulin secretion in pancreatic \(\beta\) cells. Upon glucose entry into \(\beta\) cells, glucokinase initiates glucose metabolism to increase the cytosolic ATP/ADP ratio (1). Increase in the ATP/ADP ratio leads to closure of K\(_{\text{ATP}}\) channels and membrane depolarization, which in turn opens voltage-gated calcium channels and increases of intracellular calcium, triggering insulin secretion (2). Neuronal and hormonal signals modulate secretion in response to nutrients by modifying the activity and effects of second messengers or effector molecules that control secretion (3-5).

Heterotrimeric G protein (G\(\alpha\beta\gamma\)) coupled receptors (GPCR) are the major mediators of hormonal and neuronal signals in modulating insulin secretion (6; 7). Neurotransmitters or neuro-peptides bind their respective receptors to activate the G proteins, which subsequently transmit regulatory signals by modifying the production of second messengers or interacting with effector molecules. All G protein subunits can transmit signals (8), with G\(\alpha\) being the major determinant of the specificity and strength of signaling (8; 9). There are four sub-families of G\(\alpha\) proteins (G\(\alpha_s\), G\(\alpha_q/11\), G\(\alpha_{12/13}\), and G\(\alpha_i/o\)). All of these sub-family members are expressed in \(\beta\) cells and are thought to be involved in insulin secretion regulation. For example, cholecystokinin, glucagon, glucagon-like peptide-1, and PACAP activate G\(\alpha_s\) to stimulate cAMP production and potentiate insulin secretion through Protein Kinase A-dependent and independent (i.e. cAMP-GEFII) pathways. In contrast, galanin, somatostatin, and adrenaline activate G\(\alpha_i/o\) proteins to inhibit insulin secretion through both calcium dependent and independent processes (10). The presence of these
different mechanisms highlights the diverse roles and functions of G proteins in regulating insulin secretion.

The collective roles of \( \text{G}^\alpha i/o \) proteins in insulin secretion have long been established. Pertussis toxin (or islet-activating protein, PTX) ADP-ribosylates \( \text{G}^\alpha i/o \) proteins to release the inhibitory effect of adrenaline on insulin secretion through \( \text{G}^\alpha i/o \)-coupled receptors (11-13). However, because PTX modifies \( \text{G}^\alpha i1, \text{G}^\alpha i2, \text{G}^\alpha i3, \text{and G}^\alpha o \) simultaneously, the individual in vivo function of each of these G proteins is not clear; whether they function through a common mechanism is also unclear (14).

\( \text{G}^\alpha o \), the most abundant G protein in neuronal and neuroendocrine cells, produces two protein isoforms: \( \text{G}^\alpha o 1 \) and \( \text{G}^\alpha o 2 \), through two alternatively spliced mRNAs (15; 16). The in vivo inhibitory mechanism of \( \text{G}^\alpha o \) on insulin secretion remains largely unclear due to the possible redundancy among the \( \text{G}^\alpha i/o \) proteins as well as a lack of loss-of-function studies in vivo. One possible mechanism is that \( \text{G}^\alpha o \) regulates vesicle docking or the vesicle/cytoplasmic membrane fusion process. This above hypothesis is in line with some recent findings that show the \( \text{G}^\beta \gamma \) complex can directly interact with the SNARE complex in neuroendocrine cells (17-19) to modulate secretion. Whether \( \text{G}^\alpha o \) inhibits insulin secretion through such a mechanism (e.g. \( \text{G}^\alpha o \) regulates the intracellular \( \text{G}^\beta \gamma \) concentration by sequestration in response to hormone stimulation) has not been investigated as of yet.

\( \text{G}^\alpha o^-/- \) null mice displayed severe physiological defects such as compromised viability, shortened life-span, reduced body weight, defects in pain perception, and defects in movement (tremors and seizures) (20; 21). Thus, characterization of their islet phenotype was hindered by these pleiotropic defects. Here, we utilized tissue specific loss-of-function in the mouse to analyze the function of \( \text{G}^\alpha o \) specifically in islet cells. We show that \( \text{G}^\alpha o \) deficient \( \beta \) cells have a significant increase in cell membrane-docked insulin vesicles as compared to control cells. These findings suggest that \( \text{G}^\alpha o \) functions as a repressor of insulin secretion by delaying the vesicle docking/priming process, either directly or indirectly, in \( \beta \) cells.

**RESEARCH DESIGN AND METHODS**

The \( \text{Pdx1}^{\text{Cre}} \) allele was described previously (22). The derivation of \( \text{G}^\alpha o^f \) will be described elsewhere (Jiang and Birnbaumer, Unpublished data).

Immunofluorescence/immunohistochemistry and RT-PCR followed established protocols. Mouse anti-\( \text{G}^\alpha o \) was a gift from R. Jahn (23). Guinea pig anti-insulin, guinea pig anti-glucagon, guinea pig anti-PP, and rabbit anti-SS were obtained from Dako, Carpinteria, CA. Mouse monoclonal anti-insulin antibody was purchased from Sigma-Aldrich, St. Louis, MO. Secondary antibodies used were: FITC- conjugated donkey anti-rabbit IgG; FITC-conjugated donkey anti-guinea pig IgG, and Cy3-conjugated donkey anti-mouse IgG (Jackson Immunoresearch, West Grove, PA). Goat anti-mouse IgG conjugated to Alexa Fluor 488 was from Invitrogen, Carlsbad, CA. All antibodies were used at a 1:500-1:2000 dilution. Oligos utilized for \( \text{G}^\alpha o \) RT-PCR are (see Figure 2): P1: 5'-cactgacggagcatcctccga-3'; P2: 5'-catctcaacgagtgatatcaacagctct-3'; P3: 5'-cttcctcaacaaagacattc-3'; P4: 5'-gtcgaggggctttttgtgtct-3'; P5: 5'-caagtgtgtcagcagcactca-3'; P6: 5'-cticggaggtgagccacagct-3'. Oligos utilized for insulin expression assays: 5'-cagcaagcaggtcattgttt-3' and 5'-gggaccacaaaaggaatc-3'.

For \( \beta \) cell mass assay, pancreata were weighed and cut as 20 \( \mu \)m frozen sections. One-tenth of the sections were randomly collected and stained for insulin expression. Confocal images (covering ~1/5 of all stained
pancreatic areas) were randomly captured utilizing a 5X objective and analyzed with Bioquant software (24) to calculate the area ratio between β cells and the whole pancreas to calculate islet mass.

Intraperitoneal PTX injection (at 1µg/100g body weight), IPGTT, insulin sensitivity assays, and serum insulin assays followed published procedures (25; 26). Islet isolation and perfusion followed an established protocol (27). For cAMP assays, 12 animals of each genotype were used to prepare 8 independent batches of islets. Islets were incubated in Ringer’s solution (with 2 mM glucose) with 0.1 mM IBMX for 2 hours and then used for assaying cAMP levels with the cAMP Biotrak™ enzyme immunoassay system (GE Healthcare).

For perifusion, islets of similar size and shape were utilized. Hand-picked islet cells were isolated and placed in a one-mL perfusion chamber, equilibrated in 5.6mM glucose for 30 minutes and then challenged with 16.7 mM glucose, (16.7 mM glucose + 100 µM IBMX), 300 µM tolbutamide, and 20 mM KCl. The perifusion fractions were collected in three-minute intervals at one mL/min flow rate and assayed for insulin by radioimmunoassay.

Fluorescent images were obtained using confocal microscopy (Zeiss LSM 510 inverted microscope). For transmission EM (TEM), islets were first isolated from 6 animals and fixed (0.25% gluteraldehyde in PBS) for sectioning and imaging. For quantification of vesicles docked on the cell membrane, images were captured using TEM at 10,000-15,000X magnification. The number of docked vesicles was counted before genotype identification, with vesicles whose outer surface were within 10 nm of the plasma membrane as docked granules. At least 50 randomly captured microscopic fields (from different β cells) of each genotype were analyzed before identifying their genotype. For calcium imaging, a series of images were acquired from isolated islets under glucose levels of 2, 4, 6, 8, 10 & 15 mM. Images were background subtracted and the mean Fluo-4:Fura-Red intensity ratio was calculated across the whole islet (28). This ratio was then normalized to the ratio calculated at 4 mM glucose stimulation.

Total internal reflection fluorescence microscopy (TIRFM)-based live cell imaging followed published procedures (29; 30). Briefly, islets were isolated and dispersed in calcium-free Krebs Ringer Buffer (KRB) containing 1 mM EGTA, and cultured on high refractive index cover glass (Olympus) in RPMI medium. β cells were then infected with recombinant adenovirus Adex1CA insulin-GFP (29) for observation in KRB containing 2.2 mM glucose (37°C). The Olympus TIRFM system was used with a high-aperture objective lens to observe the fluorescence of GFP with a CCD camera at 300-ms intervals using Metamorph version 7.1 (Universal Imaging). Stimulation with glucose was achieved by the addition of 52 mM glucose-KRB into the chamber for a final concentration of 22 mM glucose. Diiodomethane sulfur immersion oil (n=1.81, Cargille Laboratories) was used to make contact between the objective lens and the high refractive index cover glass. Light propagates through the cover glass at an angle of 65° and undergoes total internal reflection at the glass-cell interface. The refractive indices for the glass (n=1.8 at 488 nm) and cells (n=1.37) predict an evanescent field declining e-fold within 44 nm from the interface, and to ~10% within 100 nm. Most analyses, including tracking (single projection of different images) and area calculations were performed using Metamorph software, added with manual event selection. In this evanescent field setting, a granule would have a vertical distance of 9.6 nm from the plasma membrane and qualify as a morphologically docked granule (granule distance from plasma membrane <10 nm in electron microscopic
Gαo regulates insulin vesicle docking

We immunostained endogenous insulin granules in fixed pancreatic beta cells. Then, we manually counted bright spots as the docked granules.

All statistical analyses utilized the student’s T-test. A p-value of 0.05 or smaller was considered statistically significant. Quantified data are presented as mean ± SE.

RESULTS

Gαo is expressed in all endocrine islet cells.
We examined Gαo protein expression in both embryonic and adult pancreata using a monoclonal antibody that recognized both Gαo 1 and Gαo 2. Robust Gαo production is detected in all hormone-expressing cells in all stages examined, including E11.5, E17.5 and 3-month-old adults (Figure 1). We do not detect Gαo in exocrine acinar or pancreatic duct cells (Figure 1). Further RT-PCR analyses showed that both Gαo 1 and Gαo 2 mRNA could be detected in adult islet cells, suggesting that both isoforms might be involved in islet cell function (Figure 2A,B).

Gαo is not required for endocrine islet cell differentiation.
We utilized a Gαo conditional allele, in which two LoxP sites flanked the fifth and sixth exons of Gαo, common to Gαo 1 and Gαo 2 (GαoF, Figure 2A), to examine its role in β cell function. Deletion of the flanked exons produces a truncated mRNA that only codes for the N-terminal 156 amino acids, which lacks all motifs that bind to adenyl cyclase, PLCs, and the βγ subunits. We expect that this above manipulation results in a null Gαo allele (Gαo−). Indeed, Gαo−/− animals display identical phenotypes as previously reported for null mutants (data not shown), whereas Gαo+/− mice showed a similar phenotype as wild type littermates. Furthermore, the truncated protein did not perturb insulin secretion in a cultured β cell line (Supplementary Figure 1 in the online appendix available at http://diabetes.diabetesjournals.org).

GαoF/F; Pdx1Cre (F/F; Cre) adult animals were derived from standard genetic crosses. Pdx1Cre animals express Cre in all undifferentiated pancreatic progenitors and inactivate Gαo in all pancreatic progenitor cells of F/F; Cre mice. This allows us to examine whether Gαo plays a role in islet cell development. In addition, no Cre toxicity in Pdx1Cre animals has been observed (22). RT-PCR assays showed that the mRNA sequence corresponding to the 5th and 6th exons of Gαo was no longer detectable in islets of 4-month-old F/F; Cre animals (Figure 2B), confirming the effectiveness of Pdx1Cre for GαoF deletion.

The F/F; Cre animals were no different in body weight from their control littermates (GαoF/F or F/F) at all ages examined: 6, 9, 12 and 20 weeks (Figure 2C). No structural or behavioral (aggression, feeding, moving, and mating) defects were obvious in these animals as well. At post-natal day 1 (P1), the insulin contents in F/F; Cre and F/F pancreata were not significantly different (Supplementary Figure 2A), suggesting that Gαo is not required for β cell differentiation. By P28, the insulin content in F/F; Cre animals was reduced by 20% over that of control littermates (Figure 2D). At P56 (8 weeks), the insulin content of F/F; Cre animals had a 38% reduction compared to control littermates (Figure 2D). Consistent with this finding, the β cell mass was reduced in F/F; Cre animals at P56 as well (Figure 2E-G). The reason for this reduction in insulin content is not currently clear.

We analyzed islet morphology and expression of several genes that are required for endocrine islet cell differentiation and function, including MafA, MafB, Myt1, Nkx6.1, and Pdx1 (25) by immunofluorescence. None of the above markers were affected by Gαo inactivation (Supplementary Figure 2B and data not shown). These data suggest that Gαo is not required for islet neogenesis, even though it is
expressed in early Ngn3-expressing endocrine progenitor cells (32).

**Gαo is the major mediator of PTX’s effect on insulin secretion inhibition.** Gαi and Gαo inactivation by PTX uncouples the inhibitory effects of some neural hormones, such as adrenaline, on insulin secretion (12). Because both Gαi and Gαo are expressed in islet cells and they both can be ADP-ribosylated by PTX (14; 33; 34), it is not clear which G protein is mediating the PTX effect on insulin secretion. We utilized the Gαo mutant allele to directly investigate this question.

The fasting blood glucose levels in F/F; Cre and F/F animals were similar (Figure 3A, note the data points at “0” minutes). However, intraperitoneal glucose tolerance tests (IPGTT) showed that F/F; Cre animals have significantly improved glucose clearance over control littermates (Figure 3A). Consistent with this observation, the fasting serum insulin levels are similar between F/F and F/F; Cre animals. Fifteen minutes after glucose challenge, the serum insulin levels in F/F control animals increased by two-fold, but increased up to 10-fold in F/F; Cre mice (Figure 3B). Because the insulin sensitivity in F/F; Cre and control animals was similar (Figure 3C), the above findings demonstrate that losing Gαo potentiates insulin secretion from β-cells. We next tested whether Gαi proteins function to repress insulin secretion in the absence of Gαo. If they do, we expect that PTX treatment of F/F; Cre animals would further potentiate insulin secretion. PTX injection into F/F animals resulted in a significant increase in glucose tolerance. Whereas PTX injection into F/F; Cre animals had no significant effect (Figure 3D), suggesting that although Gαi proteins are expressed in islet cells and may be ADP-ribosylated by PTX, Gαo is the major mediator of PTX’s effect on insulin secretion.

**Gαo regulates insulin secretion at steps shared by different secretagogues.** Islet perifusion assays were utilized to directly test how Gαo inactivation affects insulin secretion in vitro. Islets from two-month-old animals were assayed for insulin secretion in response to glucose, IBMX, tolbutamide, and KCl. Glucose induces insulin secretion through metabolism to alter the ATP/ADP ratio and other metabolites. IBMX inhibits cAMP phosphodiesterase to upregulate the levels of cAMP, which activates Protein Kinase A and/or GEFII to facilitate insulin vesicle exocytosis (35; 36). Tolbutamide, a K<sub>ATP</sub> channel blocker, depolarizes β cell membrane potential, as does KCl. In response to these stimuli, the insulin secretion in the F/F; Cre islets was substantially increased compared to that of control littermates at every time point examined (Figure 4A). The biggest increase was in response to glucose, increasing as much as 369% (Figure 4B). This secretion increase was lower than what was detected during in vivo glucose challenge (Figure 3B), likely due to the synergetic effect of multiple hormones that regulate insulin secretion through Gαo in vivo, but not in vitro. Importantly, these data suggest that Gαo regulates insulin secretion through a mechanism that is shared by all these stimuli, most likely in steps that are distal to Ca<sup>2+</sup> mobilization, as suggested for in vitro-based studies (10).

Indeed, inactivation of Gαo did not significantly affect cAMP production in isolated islets (Figure 4C). In addition, similar increases in intracellular free calcium ([Ca<sup>2+</sup>]<sub>i</sub>) concentration were seen in both F/F; Cre mutant and control islets following elevated glucose stimulation (Figure 5). Synchronous bursting and spiking activity were observed at 8, 10 & 15 mM glucose respectively (Figure 5A and data not shown) in both sets of islets. The mean fold increase in [Ca<sup>2+</sup>]<sub>i</sub> was also similar for varying levels of glucose stimulation for both mutant and wild-type
islets (Figure 5B). This result suggests that Goα has little effect on β cell electrical activity and suggests that Goα regulates insulin secretion downstream of elevated [Ca^{2+}], in vivo.

Goα inhibits insulin granule docking to the β cell plasma membrane. We next examined how Goα affects insulin secretion. Because insulin vesicle docking on the cell membrane is necessary for insulin secretion, we utilized TEM to investigate whether vesicle distribution in β cells is affected by loss of Goα. The size of each vesicle in β cells did not vary between F/F; Cre and F/F control islets (Figure 6A and B). The density of granules in the F/F; Cre and control β cell remained unchanged as well (Figure 6E). However, the number of secretory vesicles in direct contact with cell membrane increased by about 100% in F/F; Cre β cells as compared to that of controls (Figure 6C, D, and F). Because TEM only allows us to examine vesicle docking on a thin section with limited depth, we utilized TIRFM to verify the above findings. TIRFM utilizes evanescent light waves to selectively illuminate the β cell surface at a 100nm depth. Thus, this technique allows us to exclusively visualize the granules which localize in the proximity of the cell membrane on a wide cell surface area. Isolated islet cells were fixed and stained with insulin antibodies and subjected to TIRFM (Figure 6G). Consistent with the above TEM-based finding, we observed a significant (P<0.01) increase in the number of insulin vesicles close to plasma membrane in Goα mutants (257/µm^2) over that of the control cells (190/µm^2) (Figure 6F). Note that the fold increase of docked vesicles revealed by TIRFM (a 35% increase) is lower than that observed from TEM-based analysis (100% increase). This is an expected result because TEM identifies the vesicles that directly contact the plasma membrane, which is only a small portion of the vesicles that localize within 100nm of the plasma membrane visualized through TIRFM. Additionally, our vesicle density count with EM and TIRM displayed a 2-fold difference (Figure 6E and H). This discrepancy could be due to the unequal vesicle distribution within the cytoplasmic compartment and cell membrane. Alternatively, it is possible that TIRFM only visualizes high-insulin content vesicles (due to the staining-related issues), whereas EM allows us to visualize all vesicles.

Goα inactivation expedites vesicle release in β cells but does not affect vesicle trafficking from cytoplasm to plasma membrane. TIRFM visualize vesicle movement in vivo in real time. We therefore recorded the vesicular dynamics close to the β cell membrane in wild type and Goα mutant animals. Dissociated β cells were transfected with retroviral particles that expressed a human insulin-EGFP fusion protein, which was previously shown to be packaged in normal insulin vesicles and not interfere with insulin trafficking. As a result, the EGFP-marked insulin vesicles could be followed in real time (29; 37).

Islet cells were stimulated with 22 mM glucose (see M&M). Vesicular movements close to the β cell membrane were recorded at 300-ms intervals with TIRFM. The number of fusion events at the plasma membrane was counted at one-minute intervals. Consistent with the perifusion assays (Figure 4), Goα mutant β cells release significantly more vesicles than control β cells (Figure 7A). In this regard, it is possible that Goα inactivation could either shorten vesicle residence time on the plasma membrane before fusion or expedite transportation of vesicles from cytoplasm to plasma membrane. In order to differentiate between these possibilities, we counted the fusion events from pre-docked vesicles and newly arrived vesicles (newcomers or vesicles that appear close to cell membranes after the start of recording) during stimulation.
Membrane-docked vesicles in \( G\alpha_0 \) mutant \( \beta \) cells showed a trend of increased readiness for release (Figure 7B). Specifically, upon glucose stimulation, 23.1\% of pre-docked insulin vesicles were released within ten minutes in control \( \beta \) cells, whereas 35.7\% of pre-docked vesicles were released within the same time frame in \( \beta \) cells without \( G\alpha_0 \) (Figure 7B); this represents a 52\% increase. On the contrary, the fusion events contributed by newly arrived vesicles did not display a significant difference between the control and mutant \( \beta \) cells (Figure 7C, 174±58 vs. 213±68; less than 23\% difference). Overall, these data suggest that one of the possible \( G\alpha_0 \) functions is to facilitate vesicle docking and, to a lesser extent, increase the readiness of vesicle fusion to the plasma membrane (Figure 7D).

**DISCUSSION**

Although the role of \( G\alpha_0 \) in insulin secretion has been implicated for half a century from PTX-based G protein uncoupling studies (11-13), the non-specificity of PTX (which inactivates both \( G\alpha_i \) and \( G\alpha_0 \)) has made it impossible to investigate how \( G\alpha_0 \) functions *in vivo*. Our findings suggest that \( G\alpha_0 \) might regulate insulin granule dynamics distal to Ca\( ^{2+} \) mobilization *in vivo*, a conclusion drawn from cell culture-based studies (38-42).

Vesicle docking is an essential step for insulin secretion. Each \( \beta \) cell contains more than 10,000 vesicles (43; 44), yet only a small portion of these vesicles can be readily released within the first phase of glucose induction (less than 10 minutes in all studied species) (2; 7). Subsequently, insulin vesicles are transported from cytoplasm to the plasma membrane for docking, priming, and fusion to sustain the second phase of release. Thus, vesicle docking, although not the rate limiting step for insulin secretion, likely plays an essential role in regulating insulin secretion. Consistent with this hypothesis, adult \( \beta \) cells that have lost the transcription factor gene FoxA2 have more insulin vesicles docked on the cell membrane and this phenotype is accompanied by excessive glucose stimulated insulin secretion (45). Thus, understanding vesicle trafficking could provide key insights into the mechanisms that regulate insulin release in response to nutritional, neuronal, and hormonal stimuli.

Both our TEM and TIRFM-based studies show that loss of \( G\alpha_0 \) results in more vesicles docking to the plasma membrane at the resting state. Furthermore, the docked vesicles in \( G\alpha_0 \) nullizygous \( \beta \) cells appear more likely to fuse with the plasma membrane than docked vesicles in control cells. These data, combine with the finding that \( G\alpha_0 \) inactivation does not significantly alter the transport of vesicles to plasma membrane, suggest that \( G\alpha_0 \) could delay vesicle docking and possibly repress vesicle priming. Further supporting this notion is our finding that \( G\alpha_0 \) does not appear to affect calcium flux, which seems to contradict some previously published findings (10). It is likely that only specific GPCR receptor-ligand coupling could affect channel activity via \( G\alpha_0 \), which cannot be activated in our *in vitro* assay. Alternatively, the *in vitro* assays may not be sensitive enough to detect the subtle channel activity alteration with or without \( G\alpha_0 \). For example, \( G\alpha_0 \) could regulate the resting Ca\( ^{2+} \) levels in \( \beta \) cells, which would be consistent with finding that resting Ca\( ^{2+} \) level affects the pool size of readily releasable granules (46). It would be interesting to analyze whether hormones, such as galanin, somatostatin, or adrenaline, can regulate specific channel activities in the presence or absence of \( G\alpha_0 \) and how this might affect the resting Ca\( ^{2+} \) levels in isolated islets.

How \( G\alpha_0 \) modulates the vesicle docking/priming process is not known. Because there are high levels of \( G\alpha_0 \) protein in neuronal and neuroendocrine cells, it was proposed that one function of \( G\alpha_0 \) was to act
Gαo regulates insulin vesicle docking as a reservoir for the Gβγ subunits within cells. When stimulated, Gαo will dissociate from the Gαβγ to release Gβγ as an effector to regulate cell function. Several lines of existing evidence support this possibility. First, expressing a Gβγ binding protein, the PH domain of the G-protein-linked receptor kinase 2, stimulates insulin secretion in response to secretagogues, similar to the consequences of Gαβγ trimer formation (47). Second, introducing Gβγ proteins in neuronal cells mimics the effect of Gαo protein activation, i.e. dissociation of the Gαβγ complex (48). In line with this possibility, loss of Gαo could reduce cellular Gβγ subunits, which results in dysregulated vesicle trafficking and secretion (17). Unfortunately, it is currently unknown which specific β or γ subunit interacts with Gαo and has thus prevented us from directly examining this possibility. Alternatively, Gαo proteins could directly interact with unknown effectors to regulate insulin secretion. Solving this issue will likely require a comprehensive understanding of all the protein/effectors that specifically interact with Gαo under normal physiological conditions. We currently do not know which possibility is likely to occur.

In summary, our analysis suggests that Gαo modulates insulin secretion by regulating vesicle docking on the β cell membrane. Addressing the specific mechanism likely requires a comprehensive analysis of proteins that interact with Gαo and how these proteins modulate vesicle trafficking, docking, priming, and fusion processes.


ACKNOWLEDGEMENTS
We thank Ethan Lee and Lindsay Bramson from Vanderbilt Medical Center, Cell and Developmental Biology, for help with preparing the manuscript. We are grateful to R. Jahn (Max Plank Institute, Munich, Germany) for providing the Gαo antibody. This research was supported by grants from the NIH (DK069771 to MJ, DK53434 to DWP), JDRF (2009-371 to GG), and by the Intramural Research Program of the NIH (Z01 ES-101643 to LB). ACP was supported by grants from the JDRF, the VA Research Service, the NIH (DK66636, DK69603, DK63439), the Vanderbilt Mouse Metabolic Phenotyping Center (DK59637), and the Vanderbilt Diabetes Research and Training Center (DK20593). Shinya Nagamatsu is supported by the following resource: KAKENHI (C-20570189, 21113523 to MOI, B-20390260 to SN), Sumitomo Foundation (to MOI), Astellas Foundation for Research on Metabolic Disorders (MOI) and Research Foundation for Opto-Science and Technology (MOI). TEM was performed in the Vanderbilt EM core facility, with help from Denny Kerns, Matt Stephenson, and Mary Dawes. The color print fee cost was covered by JDRF.

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FIGURE LEGENDS

**Figure 1. Gαo is expressed in the endocrine islet cells of the pancreas.** Expression patterns at three mouse stages, E11.5 (A), E17.5 (B-E), and 2-month-old adult (F-I), are shown. Immunofluorescence was used to visualize co-expression of Gαo with each endocrine hormone. Three panels, Gαo, hormone (green), and a merged image. Note that all hormone expressing cells express Gαo. Scale bar=20 µm.

**Figure 2. Gαo is not required for islet cell differentiation.** (A) A diagram showing the GαoF (F) allele. Only some exons are shown (from 5 to 8). Exons 7' and 8' are specific to Gαo2. Exons 7'' and 8'' are specific to Gαo1. Arrows P1-P6 indicate the oligonucleotides used for detecting Gαo mRNAs in panel (B), which shows RT-PCR detection of Gαo mRNA in 4-month-old adult islet cells. RT-reactions with insulin-specific oligos were utilized as controls (with or without reverse transcription). (P5+P6) detects Gαo1 mRNA. (P3+P4) detects Gαo2 mRNA (Cre refers to GαoF/F; *Pdx1Cre*). (P1+ P2) detects both Gαo1 and Gαo2 messages. (C)
Figure 3. Pancreas specific $G\alpha$ inactivation enhances glucose clearing capability in adult animals. (A) IPGTT results in 6 and 12 week-old animals. $G\alpha^{F/F}; Pdx1^{Cre}$ (F/F; Cre) and $G\alpha^{F/F}$ (F/F) animals were utilized. At least 13 males and 13 females of each genotype were assayed. Presented are the combined results from both males and females. (B) Serum insulin levels 0 and 15 minutes after intraperitoneal glucose injection in 12-week-old F/F; Cre and F/F males. Note the fold increases in both control (gray bars) and F/F; Cre (black bars) animals after glucose stimulation. (C) Insulin sensitivity of 12-week-old F/F; Cre and F/F animals. The “Y” axis is presented as the ratio of glucose levels between the assay point over that of the “0” minute value. (D) IPGTT results in animals treated with PTX. ($*$=P<0.01).

Figure 4. $G\alpha$ nullizygous islets secrete more insulin in response to multiple stimulations. (A) Perifusion assay results. Note the enhanced insulin secretion response to different secretagogues. IEQ= islet equivalent. ($^* = P<0.01$). (B) Total insulin release induced by different secretagogues. Data are integrated from panel A. (C) cAMP levels in mutant and control islets. The cAMP concentration is normalized against the OD280 of islet extract (as an assay of protein content).

Figure 5. Calcium responses were not altered in $G\alpha$ mutant islets. (A) Representative time courses of $[Ca^{2+}]_i$ activity estimated from the normalized Fluo4:FuraRed intensity ratio in both F/F (left) and F/F; Cre islets (right) 10 minutes after glucose challenge. Shown are data from representative islets. Each islet was monitored continuously for 2 minutes. Note that 2 mM glucose does not elicit Ca$^{2+}$ mobilization. 8 mM glucose induces synchronous bursting of Ca$^{2+}$ activity in both sets of islets. 15 mM glucose induces continuous spiking in both sets of islets. (B) Fold increases in $[Ca^{2+}]_i$ estimated from the normalized Fluo4:FuraRed intensity ratio for control islets (red) and mutant islets (blue) under varying glucose stimulation. All data is normalized to Fluo-4:Fura-Red intensity ratio at 4 mM glucose.

Figure 6. $G\alpha$ inactivation increases insulin vesicle docking to $\beta$ cell membrane. (A-D) TEM images highlighting insulin vesicle density (A and B: Note that different $\beta$ cells have different vesicle density) or membrane associated vesicles (C and D). Arrows, membrane docked vesicles. (E) Vesicle density in $\beta$ cells, presented as number of vesicles on two-dimensional views. (F) Number of vesicles docked onto cytoplasmic membrane from EM-based analysis. Data are presented as number of vesicles over length of intercellular junctions ($p<0.01$). (G) TIRFM images showing the presence of insulin vesicles on the surface of fixed and insulin antibody-stained $\beta$ cells of control (F/F) and $G\alpha$ deleted (F/F; Cre) animals. Vesicles within the circled areas were counted and presented in (H).

Figure 7. $G\alpha^{+/}$ cells release insulin vesicles more readily upon glucose stimulation. (A) The numbers of vesicle-plasma membrane fusion events at several time points with 22 mM glucose stimulation. The events are presented as fusions from pre-docked vesicles and fusions from newly arrived vesicles, respectively. (B) The % of pre-docked vesicles that are released within 10 minutes of glucose stimulation. (C) The number of newly arrived vesicles that are released.
within 10 minutes of glucose induction. (D) A simple model summarizing where Gαo could exert its function in the vesicle secretion process, including vesicle docking and possibly priming.

Figure 1
Figure 2

G_o regulates insulin vesicle docking
Figure 3

**A**

6 weeks

12 weeks

Time after glucose injection (min)

**B**

n=6

**C**

n=12

**D**

blood glucose (mg/dl)

Time after glucose injection (min)

*P<0.01

p=0.11 (n=7)
Gαo regulates insulin vesicle docking

Figure 4

Figure 5
Gαo regulates insulin vesicle docking

Figure 6

(A) and (B) show cell sections with insulin vesicles. (C) and (D) depict vesicle density and docked vesicles per mm² for different genotypes.

(E) and (F) present bar graphs comparing vesicle density and docked vesicles for F/F and F/F; Cre genotypes. The p-value for docked vesicles is p<0.01.

(G) and (H) illustrate the number of insulin vesicles per 200 mm² for F/F and F/F; Cre, with a significant difference at p<0.01.
Figure 7

A. Pre-existing pool vs. newcomer pool

B. % of pre-existing vesicles released within 5 min

C. # of newcomers released within 10 min

D. Schematic diagram of insulin vesicle docking

$\alpha$ regulates insulin vesicle docking