Gαo Represses Insulin Secretion by Reducing Vesicular Docking in Pancreatic β-Cells

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OBJECTIVE—Pertussis toxin uncoupling–based studies have shown that Gαi and Gαo can inhibit insulin secretion in pancreatic β-cells. Yet it is unclear whether Gai and Gao operate through identical mechanisms and how these G-protein–mediated signals inhibit insulin secretion in vivo. Our objective is to examine whether/how Gao regulates islet development and insulin secretion in β-cells.

RESEARCH DESIGN AND METHODS—Immunoassays were used to analyze the Gao expression in mouse pancreatic cells. Gao was specifically inactivated in pancreatic progenitor cells by pancreatic cell–specific gene deletion. Hormone expression and insulin secretion in response to different stimuli were assayed in vivo and in vitro. Electron microscope and total internal reflection fluorescence–based assays were used to evaluate how Gao regulates insulin vesicle docking and secretion in response to glucose stimulation.

RESULTS—Islet cells differentiate properly in Gao−/− mutant mice. Gao inactivation significantly enhances insulin secretion both in vivo and in isolation. Gao nullizygous β-cells contain an increased number of insulin granules docked on the cell plasma membrane, although the total number of vesicles per β-cell remains unchanged.

CONCLUSIONS—Gao is not required for endocrine islet cell differentiation, but it regulates the number of insulin vesicles docked on the β-cell membrane. Diabetes 59:2522–2529, 2010

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

Heterotrimeric G-protein (Gαβγ) coupled receptors are the major mediators of hormonal and neuronal signals in modulating insulin secretion (6,7). Neurotransmitters or neuropeptides bind their respective receptors to activate the G-proteins, which subsequently transmit regulatory signals by modifying the production of secondary messengers or interacting with effector molecules. All G-protein subunits can transmit signals (8), with Gα being the major determinant of the specificity and strength of signaling (8,9). There are four subfamilies of Gα proteins (Gαs, Gαq/11, Gα12/13, and Gαi/o). All of these subfamily members are expressed in β-cells and are thought to be involved in insulin secretion regulation. For example, cholecystokinin, glucagon, glucagon-like peptide-1, and PACAP activate Gαs to stimulate adenosine 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 Gαi/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 Gαi/o proteins in insulin secretion have long been established. Pertussis toxin (or islet-activating protein, [PTX]) ADP-ribosylates Gαi/o proteins to release the inhibitory effect of adrenaline on insulin secretion through Gαi/o-coupled receptors (11–13). However, because PTX modifies Gα1, Gα2, Gα3, and Gαo 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).

Goo, the most abundant G-protein in neuronal and neuroendocrine cells, produces two protein isoforms: Gαo1 and Gαo2, through two alternatively spliced mRNAs (15,16). The in vivo inhibitory mechanism of Gαo on insulin secretion remains largely unclear due to the possible redundancy among the Gαi/o proteins as well as a lack
of loss-of-function studies in vivo. One possible mechanism is that Gao regulates vesicle docking or the vesicle/cyttoplasmic membrane fusion process. This above hypothesis is in line with some recent findings that show the Gβγ complex can directly interact with the SNARE complex in neuroendocrine cells (17–19) to modulate secretion. Whether Gao inhibits insulin secretion through such a mechanism (e.g., Gao regulates the intracellular Gβγ concentration by sequestration in response to hormone stimulation) has not been investigated as of yet.

**Gao**+/− 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 used tissue-specific loss of function in the mouse to analyze the function of Gao specifically in islet cells. We show that Gao-deficient β-cells have a significant increase in cell membrane–docked insulin vesicles as compared with control cells. These findings suggest that Gao functions as a repressor of insulin secretion by delaying the vesicle docking/priming process, either directly or indirectly, in β-cells.

**RESULTS**

**Gao is expressed in all endocrine islet cells.** We examined Gao protein expression in both embryonic and adult pancreata using a monoclonal antibody that recognized both Gao1 and Gao2. Robust Gao production is detected in all hormone-expressing cells in all stages examined, including E11.5, E17.5, and 3-month-old adults (Fig. 1). We do not detect Gao in exocrine acinar or pancreatic duct cells (Fig. 1 and data not shown). Further RT-PCR analyses showed that both Gao1 and Gao2 mRNA could be detected in adult islet cells, suggesting that both isoforms might be involved in islet cell function (Fig. 2A and B).

**Gao is not required for endocrine islet cell differentiation.** We used a Gao conditional allele, in which two LoxP sites flanked the fifth and sixth exons of Gao, common to Gao1 and Gao2 (Gao1−/−, Fig. 2A), to examine its role in β-cell function. Deletion of the flanked exons produces a truncated mRNA that only codes for the NH2-terminal 156 amino acids, which lacks all motifs that bind to adenyl cyclase, phospholipase Cs (PLCs), and the βγ subunits. We expect that this above manipulation results in a null Gao allele (Gao−/−). Indeed, Gao−/− animals display identical phenotypes as previously reported for null mutants (data not shown), whereas Gao+/− 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 Fig. 1, available in an online appendix at http://diabetes.diabetesjournals.org/cgi/content/full/db09-1719/DC1).

**Gao**+/−,Pdx1Cre (F/F;Cre) adult animals were derived from standard genetic crosses. Pdx1Cre+/− animals express Cre in all undifferentiated pancreatic progenitors and inactivate Gao in all pancreatic progenitor cells of F/F;Cre mice. This allows us to examine whether Gao plays a role in islet cell development. In addition, no Cre toxicity in
**FIG. 1.** Gao is expressed in the endocrine islet cells of the pancreas. Expression patterns at three mouse stages, E11.5 (A), E17.5 (B–E), and 3-month-old adult (F–I), are shown. Immunofluorescence was used to visualize coexpression of Gao with each endocrine hormone. Three panels: Gao, hormone (green), and a merged image. Note that all hormone-expressing cells express Gao. Scale bar = 20 μm. PP, pancreatic polypeptide; SS, somatostatin. (A high-quality digital representation of this figure is available in the online issue.)

Pdx1<sup>Cre</sup> animals has been observed (22). RT-PCR assays showed that the mRNA sequence corresponding to the fifth and sixth exons of Gao was no longer detectable in islets of 4-month-old F/F;Cre animals (Fig. 2B), confirming the effectiveness of Pdx1<sup>Cre</sup> for Gao<sup>Cre</sup> deletion.

The F/F;Cre animals were no different in body weight from their control littermates (Gao<sup>FF</sup> or F/F) at all ages examined: 6, 9, 12, and 20 weeks (Fig. 2C). Additionally, no structural or behavioral (aggression, feeding, moving, and mating) defects were obvious in these animals. At postnatal day 1 (P1), the insulin contents in F/F;Cre and F/F pancreata were not significantly different (supplementary Fig. 2A, available in an online appendix), suggesting that Gao 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 (Fig. 2D). At P56 (8 weeks), the insulin content of F/F;Cre animals had a 38% reduction compared with control littermates (Fig. 2D). Consistent with this finding, the β-cell mass was reduced in F/F;Cre animals at P56 as well (Fig. 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 Gao inactivation (supplementary Fig. 2B and data not shown). These data suggest that Gao is not required for islet neogenesis, even though it is expressed in early Ngn3-expressing endocrine progenitor cells (32).

**Gao is the major mediator of PTX’s effect on insulin secretion inhibition.** Gao and Gao inactivation by PTX uncouples the inhibitory effects of some neural hormones, such as adrenaline, on insulin secretion (12). Because both Gao and Gao 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 used the Gao mutant allele to directly investigate this question.

The fasting blood glucose levels in F/F;Cre and F/F animals were similar (Fig. 3A, note the data points at 0 min). However, IPGTT showed that F/F;Cre animals have significantly improved glucose clearance over control littermates (Fig. 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 2-fold but increased up to 10-fold in F/F;Cre mice (Fig. 3B). Because the insulin sensitivity in F/F;Cre and control animals was similar (Fig. 3C), the above findings demonstrate that losing Gao potentiates insulin secretion from β-cells. We next tested whether Gao proteins function to repress insulin secretion in the absence of Gao. 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 (Fig. 3D), suggesting that although Gao proteins are expressed in islet cells and may be ADP-ribosylated by PTX, Gao is the major mediator of PTX’s effect on insulin secretion.

**Gao regulates insulin secretion at steps shared by different secretagogues.** Islet perfusion assays were used to directly test how Gao inactivation affects insulin secretion in vitro. Islets from 2-month-old animals were assayed for insulin secretion in response to glucose, IBMX, tolbutamide, and KCl stimulation. Glucose induces insulin secretion through metabolism to alter the ATP/ADP ratio and other metabolites. IBMX inhibits cAMP phosphodies-
terase to upregulate the levels of cAMP, which activates protein kinase A and/or GEFII to facilitate insulin vesicle exocytosis (35,36). Tolbutamide, a KATP 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 with that of control littermates at every time point examined (Fig. 4A). The biggest increase was in response to glucose, increasing as much as 369% (Fig. 4B). The density of granules in the F/F;Cre and control β-cells remained unchanged as well (Fig. 6E). However, the number of secretory vesicles in direct contact with the cell membrane increased by about 100% in F/F;Cre β-cells as compared with that of controls (Fig. 6C, D, and F). Because TEM only allows us to examine vesicle docking on a thin section with limited depth, we used TIRFM to verify the above findings. TIRFM uses evanescent light waves to selectively illuminate the β-cell surface at a 100-nm depth. Thus, this technique allows us to exclusively visualize the granules that 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 (Fig. 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 Gao mutants (257/μm²) over that of the control cells (190/μm²) (Fig. 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 be-

![Diagram](https://example.com/diagram.png)

**FIG. 4.** Gao 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 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).
cause TEM identifies the vesicles that directly contact the plasma membrane, which is only a small portion of the vesicles that localize within 100 nm of the plasma membrane visualized through TIRFM. Additionally, our vesicle density count with EM and TIRFM displayed a twofold difference (Fig. 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 antibody staining–related issues), whereas EM allows us to visualize all vesicles.

**Gao inactivation expedites vesicle release in β-cells but does not affect vesicle trafficking from cytoplasm to plasma membrane.** TIRFM visualizes vesicle movement in vivo in real time. We therefore recorded the vesicular dynamics close to the β-cell membrane in wild-type and Gao mutant animals. Dissociated β-cells were transfected with retroviral particles that expressed a human insulin–enhanced green fluorescent protein (EGFP) fusion protein, which was previously shown to be packaged in normal insulin vesicles and to 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 mmol/l glucose (see RESEARCH DESIGN AND METHODS). Vesicular movements close to the β-cell membrane were recorded at 300-ns intervals with TIRFM. The number of fusion events at the plasma membrane was counted at 1-min intervals. Consistent with the perfusion assays (Fig. 4), Gao mutant β-cells release significantly more vesicles than control β-cells (Fig. 7A). In this regard, it is possible that Gao 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 predocked 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 Gao mutant β-cells showed a trend of increased readiness for release (Fig. 7B). Specifically, upon glucose stimulation, 23.1% of predocked insulin vesicles were released within 10 min in control β-cells, whereas 35.7% of predocked vesicles were released within
the same time frame in β-cells without Gao (Fig. 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 β-cells (Fig. 7C; 174 ± 58 vs. 213 ± 68; <23% difference). Overall, these data suggest that one of the possible Gao functions is to facilitate vesicle docking and, to a lesser extent, to increase the readiness of vesicle fusion to the plasma membrane (Fig. 7D).

DISCUSSION

Although the role of Gao in insulin secretion has been implicated for one-half century from PTX-based G-protein uncoupling studies (11–13), the nonspecificity of PTX (which inactivates both Goi and Gao) has made it impossible to investigate how Gao functions in vivo. Our findings suggest that Gao might regulate insulin granule dynamics distal to Ca²⁺ mobilization in vivo, a conclusion drawn from cell culture–based studies (38–42).

Vesicle docking is an essential step for insulin secretion. Each β-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 (<10 min 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 β-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 Gao results in more vesicles docking to the plasma membrane at the resting state. Furthermore, the docked vesicles in Gao nullizygous β-cells appear more likely to fuse with the plasma membrane than docked vesicles in control cells. These data, combined with the finding that Gao inactivation does not significantly alter the transport of vesicles to plasma membrane, suggest that Gao could delay vesicle docking and possibly repress vesicle priming. Further supporting this notion is our finding that Gao does not appear to affect calcium flux, which seems to contradict some previously published findings (10). It is likely that only specific G-protein (Goβγ)-coupled receptor–ligand coupling could affect channel activity via Gao, 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 Gao. For example, Gao could regulate the resting Ca²⁺ levels in β-cells, which would be consistent with the finding that resting Ca²⁺ 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 Gao and how this might affect the resting Ca²⁺ levels in isolated islets.

How Gao modulates the vesicle docking/priming process is not known. Because there are high levels of Gao protein in neuronal and neuroendocrine cells, it was proposed that one function of Gao was to act as a reservoir for the Gβγ subunits within cells. When stimulated, Gao 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 Gao protein activation, that is, dissociation of the Gαβγ complex (48). In line with this possibility, loss of Gao 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 Gao and has thus prevented us from directly examining this possibility. Alternatively, Gao 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 Gao under normal physiological conditions. We currently do not know which possibility is likely to occur.

In summary, our analysis suggests that Gao 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 G0 and how these proteins modulate vesicle trafficking, docking, priming, and fusion processes.

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