Augmented glucose-induced insulin release in mice lacking G_{o2}, but not G_{o1} or G_i proteins

Ying Wang[a], Sangeun Park[a], Neil S. Bajpayee[a], Yoshiko Nagaoka[a], Guylain Boulay[b,1], Lutz Birnbaumer[b,2], and Meisheng Jiang[a,2]

[a]Department of Molecular and Medical Pharmacology, David Geffen School of Medicine, University of California, Los Angeles, CA 90095; and [b]Laboratory of Neurobiology, Division of Intramural Research, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709

Contributed by Lutz Birnbaumer, December 17, 2010 (sent for review November 23, 2010)

Insulin secretion by pancreatic β cells is a complex and highly regulated process. Disruption of this process can lead to diabetes mellitus. One of the various pathways involved in the regulation of insulin secretion is the activation of heterotrimeric G proteins. *Bordetella pertussis* toxin (PTX) promotes insulin secretion, suggesting the involvement of one or more of three G_i and/or two G_o proteins as suppressors of insulin secretion from β cells. However, neither the mechanism of this inhibitory modulation of insulin secretion nor the identity of the $G_{i/o}$ proteins involved has been elucidated. Here we show that one of the two splice variants of G_o, G_{o2}, is a key player in the control of glucose-induced insulin secretion by β cells. Mice lacking $G_{o2}\alpha$, but not those lacking α subunits of either G_{o1} or any G_i proteins, handle glucose loads more efficiently than wild-type (WT) mice, and do so by increased glucose-induced insulin secretion. We thus provide unique genetic evidence that the G_{o2} protein is a transducer in an inhibitory pathway that prevents damaging oversecretion of insulin.

somatostatin | exocytosis | islet activating protein | signal transduction | G-protein-coupled receptor

Diabetes mellitus is characterized by abnormalities in insulin secretion that may be either a primary defect, as seen in type I diabetes, or a secondary defect, where secretion is inadequate to overcome primary insulin resistance seen in type II diabetes. In either case, individuals are hyperglycemic. Insulin is the master controller of glucose metabolism, and its release from β cells is tightly regulated. Many factors, including hormones, neuropeptides, and neurotransmitters regulate insulin secretion by activating heterotrimeric G proteins, which can control the output of insulin in response to physiological demands (1, 2). Activation of pathways mediated by G_s and/or $G_{q/11}$ stimulates insulin release from β cells (3, 4). The involvement of G_i/G_o proteins as inhibitors of insulin secretion from β cells was originally uncovered in studies on the *Bordetella pertussis* toxin (PTX) in animals and cells (5, 6). Previously called islet-activating protein (IAP), PTX was shown to lower glucose levels in the bloodstream by increasing insulin secretion from β cells (7). The enhanced secretion resulted from the removal of tonic inhibition exerted by neurotransmitters/hormones, including adrenaline (8), galanin (9), and ghrelin (10). PTX catalyzes the ADP ribosylation of a carboxyl-terminal cysteine present in the α subunits of the G protein subgroup now referred to as G_i/G_o (11). This event causes these G proteins to become uncoupled from receptors and thereby disrupts the signal transduction process. The nonsensory PTX-sensitive G_i/G_o G proteins encompass three G_i's (G_{i1}, G_{i2}, and G_{i3}), and two G_o's (G_{o1} and G_{o2}). The α subunits of G_i and G_o display extensive homology and are functionally similar as they can be activated by the same or similar receptors and appear to signal to partially overlapping sets of effectors (12). This has raised questions whether the individual G_i and G_o proteins function distinctively between different receptors and effectors or whether they are simply isoforms of one another. To address these questions and to better define the functional role of G_o/G_i proteins in vivo, we have generated knockout animals deficient in each of the α subunits of the G_i/G_o group (13–15). Analysis of these mutant animals reveals that each knockout has unique deficiencies or abnormalities, suggesting that each member mediates some distinct biological functions. Like G_s, all three G_i proteins are expressed in most cells in the body. G_o has a less ubiquitous expression pattern than G_i or G_s and is highly expressed in the central and peripheral nervous systems, where it was originally identified (16, 17). G_o is also expressed in the heart and in the endocrine system, including the pituitary gland and pancreatic islets (18, 19).

Insulin secretion can be modulated through cAMP-dependent and -independent pathways (1, 20). Activation of G_s in pancreatic β cells enhances insulin release through an adenylyl cyclase/cAMP-mediated mechanism. All G_i/G_o proteins serve as substrates for PTX-mediated ADP ribosylation, which blocks inhibition of adenylyl cyclase in β cells mediated by G_i proteins (21). However, G_o proteins do not mediate inhibition of adenylyl cyclase in vitro (22). Moreover, the inhibitory hormones, galanin and ghrelin, can block insulin secretion stimulated by potassium-induced depolarization (9, 10); somatostatin, galanin, and epinephrine inhibit Ca^{2+} induced insulin release in permeabilized cells (23–25), suggesting adenylyl cyclase/cAMP-independent regulation of insulin secretion exists. We recently reported that G_o is required for normal glucose-regulated insulin secretion. Conditional disruption of both G_o isoforms in islets results in reduction of readily releasable pools of insulin (26). However, to date, the precise G protein(s) responsible for the inhibition of insulin secretion has not been identified. In this study, using animal models, we demonstrate that among the five nonsensory PTX substrates (G_{i1}, G_{i2}, G_{i3}, G_{o1}, and G_{o2}), G_{o2} is the target of PTX responsible for the increased insulin release and therefore a key physiological player in the control of glucose-induced insulin secretion by β cells.

Results and Discussion

As an initial step to identify the G protein involved in tonic inhibition of insulin release, we examined the expression of $G_{i/o}$ proteins and verified their absence in the corresponding knockout mice by examining PTX-mediated [^{32}P]ADP ribosylation of G_i/G_o proteins from isolated islets. The $G_{o1}\alpha$ and $G_{o2}\alpha$ isoforms of $G_o\alpha$ were found in pancreatic islets, but were not detectable in acinar cells (Fig. 1*A*), and signals corresponding to either $G_{o1}\alpha$ or $G_{o2}\alpha$ are absent from the islets isolated from $G_o\alpha$ knockout mice. In contrast to $G_o\alpha$, the radio-labeled protein bands that correspond to $G_{i1}/G_{i3}\alpha$ and $G_{i2}\alpha$ are present in both pancreatic islets and acinar cells. As was the case for the α subunits of G_{o1}/G_{o2}, α subunits for G_{i1}, G_{i2}, and G_{i3} were absent in the corresponding knockout mice (15, 27). We noted no significant change in the expression of G_o proteins in pancreatic islets from G_i-deficient mice.

To delineate the G_i/G_o subtype responsible for the inhibitory regulation of insulin secretion, we performed glucose tolerance

Author contributions: Y.W. and M.J. designed research; Y.W., S.P., N.S.B., Y.N., G.B., and M.J. performed research; L.B. and M.J. analyzed data; and L.B. and M.J. wrote the paper.

The authors declare no conflict of interest.

[1]Present address: Department of Pharmacology, University of Sherbrooke, QC, Canada J1H 5N4.

[2]To whom correspondence may be addressed. E-mail: birnbau1@niehs.nih.gov or jm@ucla.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1018903108/-/DCSupplemental.

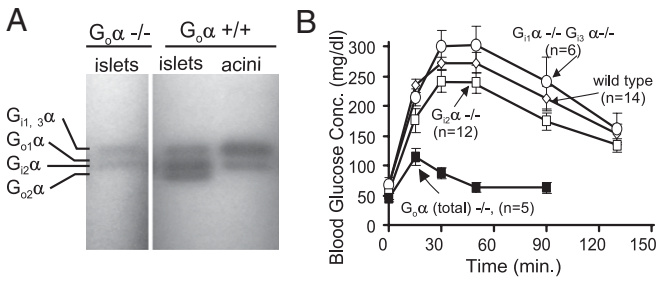

Fig. 1. (*A*) $G_o\alpha$ proteins are absent in pancreatic islets of $G_o\alpha^{-/-}$ mice; disruption of $G_o\alpha$ expression does not significantly alter expression of $G_i\alpha$ proteins. Homogenates of islets and acinar cells from $G_o\alpha^{-/-}$ and wild-type mice were ADP ribosylated by pertussis toxin with [^{32}P]ADP ribose. Proteins from a matched number of islets or acinar cells were resolved by SDS–urea PAGE. The labeled proteins were visualized by autoradiography. (*B*) "Improved" glucose tolerance tests in $G_o\alpha^{-/-}$ but not in $G_i\alpha$-deficient mice. Overnight-fasted mice received an i.p. injection of 1.5 mg of glucose per gram of bodyweight. Blood glucose levels were measured at the indicated times. Glucose levels are means ± SEM; *n* = number of mice tested.

tests in $G_o\alpha$ and $G_i\alpha$ knockout mice and in age-matched wild-type (WT) controls. Mice deficient in $G_o\alpha$, by conditional deletion as recently reported (26) and conventional deletion (Fig. 1*B*), were found to have altered tolerance tests. In contrast, the tolerance test is not significantly affected in G_i-deficient mice. The $G_o\alpha^{-/-}$ mice had an "improved" glucose tolerance, indicating that they can clear a glucose load more efficiently than the wild-type controls. This improved glucose tolerance phenomenon is similar to that seen in animals treated with PTX (28). These results indicate that one or both isoforms of G_o, but not the G_i proteins, are involved in normal regulation of insulin secretion.

Mice homozygous for loss of $G_o\alpha$ ($G_o\alpha^{-/-}$) are obtained from crossing heterozygotes ($G_o\alpha^{+/-}$). $G_o\alpha^{-/-}$ mice are born, but most die before they reach adulthood. The few $G_o\alpha^{-/-}$ survivors are smaller with a mean body weight of only 45% of their $G_o\alpha^{+/+}$ or $G_o\alpha^{+/-}$ littermates. Surviving $G_o\alpha^{-/-}$ mice have a generalized tremor, turning behavior, and impaired locomotion (13). These mice also lack the b-wave in electroretinograms (ERG), confirming that G_o mediates the signal transduction downstream of mGluR6 receptor in retinal ON-bipolar cells (29). The wide array of deficiencies in $G_o\alpha^{-/-}$ mice suggests that one or both of the G_o variants mediates critical biological processes in several parts of the body, including the β cells of pancreatic islets. $G_{o1}\alpha$ and $G_{o2}\alpha$ are identical throughout the first two-thirds of the protein and only differ in their last third, which is encoded in alternate exons 7 and 8 (30–32). These exons encode the last 113 amino acids of the $G_o\alpha$ proteins, which include key portions of the guanine nucleotide binding pocket and importantly, the receptor-interacting residues of the C terminus (33, 34). It has been shown that G_{o1} and G_{o2} are activated by distinct populations of receptors to regulate common biological responses (35). In our studies, $G_o\alpha$ knockout was obtained by disrupting exon 6, the last exon common for both $G_{o1}\alpha$ and $G_{o2}\alpha$ (13). To test whether one or both of the G_o proteins participates in inhibitory regulation of insulin secretion, we generated subtype-selective $G_{o1}\alpha$-specific and $G_{o2}\alpha$-specific knockout mice. $G_{o1}\alpha$ was disrupted by insertion of a neomycin cassette into exon 7-1 (the exon 7 for $G_{o1}\alpha$) using a targeting vector of the replacement type (Fig. S1). Because exons 7-2 and 8-2 (exons 7 and 8 for $G_o2\alpha$) are between exons 6 and 7-1, inactivation of the $G_{o2}\alpha$ splice variant was done with a two-step "hit and run" strategy, using an insertion-type targeting vector. This allows for introduction of point mutations into a genome (36). $G_{o2}\alpha$ was thus inactivated by creating an in-frame stop codon in exon 7-2, leaving the rest of the gene unaffected (Fig. S2) and ensuring that the $G_{o1}\alpha$ protein expression remains unaffected in $G_{o2}\alpha$ knockouts. The targeting vectors were electroporated into ES cells, neomycin-resistant clones were selected, and correct homologous recombinants with targeted $G_{o1}\alpha$ and $G_{o2}\alpha$ were identified by genomic Southern blot analysis. The counter selectable marker co-inserted into the ES cells with the targeted $G_{o2}\alpha$ gene was then removed in the "run" step that entails an intrachromosomal homologous recombination. Animals lacking $G_{o1}\alpha$ and $G_{o2}\alpha$ were derived from the targeted ES cells as described (37).

In many ways, $G_{o1}\alpha^{-/-}$ mice resemble the $G_o\alpha[total]^{-/-}$ mice described previously (13). They are born in normal numbers, but most die before reaching adulthood. The few survivors show neuronal deficiencies, including tremor, turning behavior, impairment of locomotion, and they lack ERG b-waves contributed by retinal ON-bipolar cells. $G_{o2}\alpha^{-/-}$ mice, unlike the $G_o\alpha[total]^{-/-}$ and $G_{o1}\alpha^{-/-}$ mice, have a higher survival rate. In fact, $G_{o2}\alpha$ null mice are obtained at the expected Mendelian frequency, do not differ from their wild-type littermates in overall health, fertility, and longevity, and have a normal growth rate. Histological examinations showed no abnormalities in brain, heart, liver, lung, spleen, pancreas, and intestine of $G_{o1}\alpha$ and $G_{o2}\alpha$ deficient mice. Expression of the $G_{o1}\alpha$ and $G_{o2}\alpha$ proteins was evaluated by Western blot analysis using $G_{o1}\alpha$- and $G_{o2}\alpha$-specific antibodies. Both anti-$G_{o1}\alpha$ and anti-$G_{o2}\alpha$ antibodies recognize G_{o1} and $G_{o2}\alpha$ subunits specifically, without apparent cross-reactivity (Fig. 2*A*). Each antibody recognized a ∼39-kDa protein band, which corresponds to the migration of $G_{o1}\alpha$ and $G_{o2}\alpha$ proteins (32). As expected, no $G_{o1}\alpha$ signal was detected in brain homogenates from $G_{o1}\alpha^{-/-}$ and $G_o\alpha[total]^{-/-}$ mice. Likewise, the $G_{o2}\alpha$ signal was absent in brain homogenates from $G_{o2}\alpha^{-/-}$ and $G_o\alpha[total]^{-/-}$ mice. The absence of $G_{o2}\alpha$ was also confirmed in pancreatic islets of $G_{o2}\alpha^{-/-}$ mice (Fig. 2*A*). These findings demonstrate that preventing the expression of $G_{o2}\alpha$ does not hamper expression of $G_{o1}\alpha$ or vice versa. In fact, expression of $G_{o2}\alpha$ was increased in $G_{o1}\alpha^{-/-}$ islets, and the level of $G_{o1}\alpha$ was increased in $G_{o2}\alpha^{-/-}$ samples. The selective expression of $G_o\alpha$ in the endocrine cells of the pancreas was confirmed by immunohistochemical staining of sections of wild-type mice (Fig. 2*B*). Immunostaining of pancreatic sections from $G_o\alpha$-deficient mice confirmed the absence of $G_{o1}\alpha$ and $G_{o2}\alpha$ proteins in $G_{o1}\alpha^{-/-}$ and $G_{o2}\alpha^{-/-}$ pancreatic islets, respectively. Moreover, the number and the size of islets of Langerhans were found to be comparable between $G_{o1}\alpha^{-/-}$, $G_{o2}\alpha^{-/-}$, and wild-type controls. Both G_{o2} and $G_{o1}\alpha$ proteins are present in all endocrine cells of the pancreas, but not in the exocrine cells. The subcellular localization of $G_{o2}\alpha$ and $G_{o1}\alpha$ in islet cells was similar. Staining was concentrated at plasma membranes with little or no staining in cytoplasmic or nuclear areas (Fig. S3). In contrast, anti-insulin antibody staining was localized to the cytoplasm of β cells. To confirm that $G_{o2}\alpha$ proteins are present in pancreatic endocrine cells, we performed dual-antibody staining of pancreatic sections from wild-type mice using anti-$G_{o2}\alpha$ antibodies along with anti-insulin (β cell), glucagon (α cell), somatostatin (δ cell), or pancreatic polypeptide (pp cell) antibodies (Fig. 2*C*). Cells staining positively for insulin, glucagon, somatostatin, and pancreatic polypeptide (PP) are also positive for the $G_{o2}\alpha$ protein, demonstrating that G_o is expressed in β, α, δ, and pp cells of the endocrine pancreas. These results confirm the expression of both G_{o1} and $G_{o2}\alpha$ in islet cells. Loss of $G_{o2}\alpha$ and $G_{o1}\alpha$ in mice does not appear to adversely affect islet cell proliferation or differentiation.

To examine the role of G_{o1} or G_{o2} proteins in maintaining glucose homeostasis, glucose, and insulin tolerance tests were performed on $G_{o1}\alpha^{-/-}$ and $G_{o2}\alpha^{-/-}$ mice (Fig. 3*A*). Because both $G_o\alpha$ isoforms are expressed in islet cells and serve as substrates for PTX, either isoform could be the target of islet-activation by PTX. After glucose was administrated, blood glucose levels in wild-type and $G_{o1}\alpha^{-/-}$ mice peaked within 10–20 min and then gradually decreased to basal levels after 150 min. In $G_{o2}\alpha^{-/-}$ mice, the peak value of blood glucose was significantly lower than that seen in wild-type and $G_{o1}\alpha^{-/-}$ mice. Furthermore, the decline of blood glucose was faster than in wild-type mice, showing that the phenotype observed in $G_o\alpha[total]^{-/-}$ mice is recapitulated in $G_{o2}\alpha$ knockout mice. It is noteworthy that the baseline in $G_{o2}\alpha^{-/-}$ mice during overnight fasting was also lower than that of wild type. Thus, the "improved" glucose tolerance test seen in $G_o\alpha[total]^{-/-}$ mice is associated with the loss of $G_{o2}\alpha$, not that of $G_{o1}\alpha$.

Knockdown of $G_{i2}\alpha$, another member in $G_{i/o}$ protein family, has been shown to alter insulin sensitivity of peripheral tissues (38). To test whether loss of $G_{o2}\alpha$ has a similar effect that could

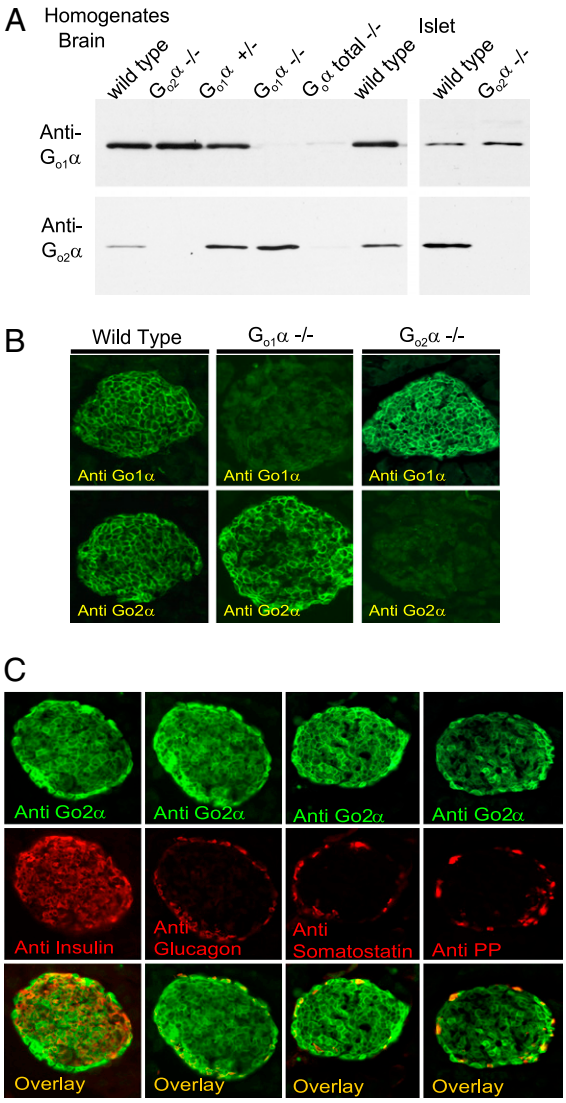

Fig. 2. Loss of $G_{o1}\alpha$ and $G_{o2}\alpha$ in the corresponding knockout mice. (*A*) Western blots analysis of brain (*Left*) and pancreatic islets (*Right*) homogenates. (*B*) Immunohistochemical localization of $G_{o1}\alpha$ and $G_{o2}\alpha$ proteins in mouse islets from wild-type, $G_{o1}\alpha$, and $G_{o2}\alpha$ knockout mice. Seven-micrometer-thick cryosections were fixed and immunostained with antibodies anti-$G_{o1}\alpha$, and anti-$G_{o2}\alpha$. (*C*) Colocalization of $G_{o2}\alpha$ with insulin, glucagon, somatostatin, and pancreatic polypeptide. WT pancreas cryosections were fixed and dual immunostained with anti-$G_{o2}\alpha$ plus anti-insulin, anti-glucagon, anti-somatostatin, and anti-PP antibodies. The immune complexes were visualized with fluorescein-labeled secondary antibodies.

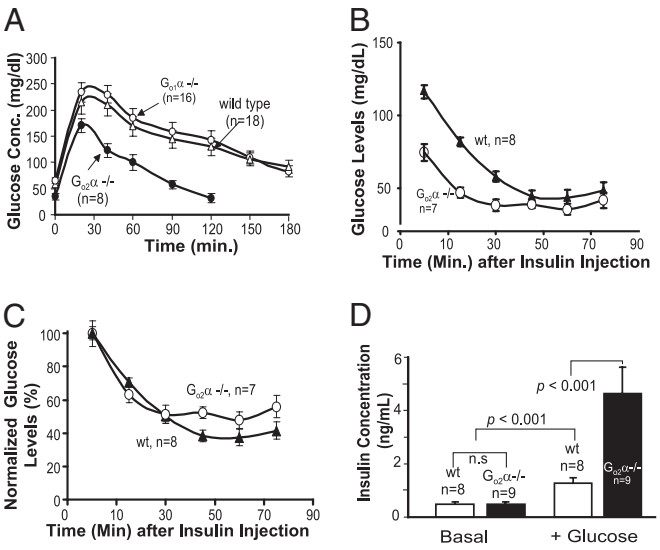

Fig. 3. Glucose and insulin tolerance tests and glucose-induced insulin secretion in $G_{o2}\alpha^{-/-}$ mice. (*A*) Glucose tolerance test in $G_{o2}\alpha^{-/-}$, $G_{o1}\alpha^{-/-}$, and WT control mice. Fasted mice received 1.5 mg of glucose per gram of bodyweight i.p. Values are expressed as means ± SEM. (*B* and *C*) Insulin tolerance tests. *B* depicts absolute blood glucose levels; *C*, normalized data expressed as the percentage of initial blood glucose levels. Blood glucose levels were measured at the indicated times following i.p. injection of 0.60 IU of insulin per kilogram of body weight. (*D*) Insulin secretion in WT and $G_{o2}\alpha^{-/-}$ mice in response to an i.p. injection of 1.5 mg of glucose per gram of bodyweight. Values are mean insulin levels (±SEM) before and 20 min after glucose administration. *n* = number of mice tested; *P*, Student's *t* test.

contribute to the altered glucose tolerance test, an insulin tolerance test was performed in $G_{o2}\alpha^{-/-}$ knockout mice. Although the initial basal glucose levels in $G_{o2}\alpha^{-/-}$ are lower than that in wild type, both groups' mice show the hypoglycemic response to exogenous insulin (Fig. 3*B*). Normalized glucose responses in $G_{o2}\alpha^{-/-}$ were indistinguishable from that of wild-type mice (Fig. 3*C*). This indicates that lack of $G_{o2}\alpha$ does not significantly alter insulin sensitivity of peripheral tissues. These observations suggest that the more effective handling of a glucose load by $G_{o2}\alpha^{-/-}$ mice is likely due to augmented insulin release from β cells. To confirm this hypothesis, $G_{o2}\alpha^{-/-}$ mice were studied for insulin secretion upon glucose administration. After overnight fasting, mice were administered glucose intraperitoneally and serum insulin levels were measured by ELISA. Basal insulin levels in control and $G_{o2}\alpha^{-/-}$ mice were similar with mean values of 0.49 ng/mL ± 0.10 (SEM, $n = 8$) and 0.50 ng/mL ± 0.09 ($n = 9$), respectively. Twenty minutes after the administration of glucose, insulin levels increased to 1.30 ng/mL ± 0.20 ($n = 8$) in control mice and to 4.70 ng/mL ± 0.98 ($n = 9$) in $G_{o2}\alpha^{-/-}$ mice (Fig. 3*D*). Thus, $G_{o2}\alpha^{-/-}$ mice respond to glucose with an enhanced pancreatic output of insulin, reducing the peak levels to which blood glucose increases after a glucose load and shortening the time required to return to basal levels.

To verify in vivo observations and provide direct evidence that the "improvement" in glucose tolerance in mice lacking the $G_{o2}\alpha$ is due to enhanced insulin secretion from β cells, we performed ex vivo perifusion assays on isolated islets and assessed the β-cell response to glucose directly. Mouse pancreata were digested with collagenase, and isolated islets were packed in perifusion chambers. Islets were first perifused with low glucose buffer to stabilize the basal levels of insulin secretion. Islets were then perifused with 16.2 mM glucose buffer for 120 min, returned to the basal condition for 30 min, followed by 30 mM KCl stimulation. Wild-type, $G_{o2}\alpha^{+/-}$, and $G_{o2}\alpha^{-/-}$ islets exhibited a first peak insulin secretion within 5–10 min, followed by a sustained second phase (Fig. 4*A*). Both the first (2–18 min) and second sustained (18–60 min) phases are greatly enhanced in $G_{o2}\alpha^{-/-}$ islets (Fig. 4 *A* and *B*). Areas under the curve of the first and second phases for wild type are 0.212 ± 0.043 (mean ± SEM) and 0.767 ± 0.137, respectively. Areas under the curve of the first and second phases for $G_{o2}\alpha^{+/-}$ are 0.212 ± 0.034 and 0.629 ± 0.063, respectively; Areas under the curve of the first and second phases for $G_{o2}\alpha^{-/-}$ are 0.715 ± 0.120 and 1.664 ± 0.217, respectively. Thus, islets from $G_{o2}\alpha^{-/-}$ mice secrete about threefold more insulin than islets from $G_{o2}\alpha^{+/-}$ heterozygous and wild-type controls in response to the same glucose stimulation. KCl-induced insulin release is also enhanced in $G_{o2}\alpha^{-/-}$ islets, and areas under the curve for wild type, $G_{o2}\alpha^{+/-}$, and $G_{o2}\alpha^{-/-}$ are 0.121 ± 0.039, 0.154 ± 0.065, and 0.365 ± 0.043, respectively. This result is consistent with our in vivo observations that $G_{o2}\alpha^{-/-}$ mice secrete more insulin upon glucose stimulation. Therefore, the observed increase in circulating insulin levels in $G_{o2}\alpha^{-/-}$ mice is likely to

PHARMACOLOGY

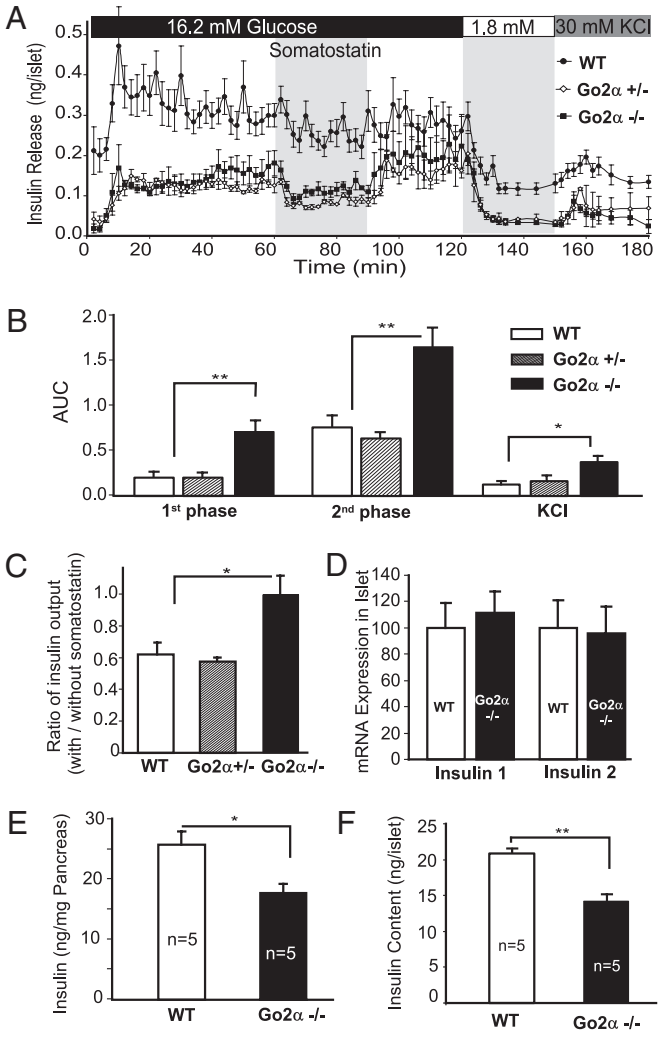

Fig. 4. Islet perifusion, insulin gene expression, and insulin contents. (*A*) Perifusion of wild-type and $G_{o2}\alpha^{-/-}$ islets. Average of three to five experiments for each genotype. (*B*) Area under curve of insulin in the first (2–18 min), sustained phases (18–60 min), and potassium-induced release (150–172 min). (*C*) Ratio of insulin output rate for the period with somatostatin (60–90 min) over the period after the removal of somatostatin (90–120 min). (*D*) mRNA expression of insulin in wild-type and $G_{o2}\alpha^{-/-}$ islets (n = 4–5). (*E*) Average insulin content in pancreas, n = 5. (*F*) Average insulin content in islets, n = 5. Data are presented as means ± SEM.

be accounted for by enhanced insulin secretion by β cells. Although we observed that there is an up-regulation of $G_{o1}\alpha$ protein in $G_{o2}\alpha^{-/-}$ and $^{+/-}$ mice, the heterozygous $G_{o2}\alpha^{+/-}$ islets exhibit the same phenotype as wild-type islets during glucose-induced insulin secretion. This suggests that the enhanced insulin release in $G_{o2}\alpha^{-/-}$ islets is not due to overexpression of $G_{o1}\alpha$; rather it is the direct consequence of loss of $G_{o2}\alpha$.

Several inhibitory hormones/neurotransmitters, such as somatostatin, galanin, and norepinephrine, elevate blood glucose levels by inhibiting insulin release. Their actions are sensitive to the PTX treatment, demonstrating that they are $G_{i/o}$ protein mediated. To test whether G_{o2} mediates the neurohormonal inhibitory effect on insulin release, we chose to test somatostatin using the islet perifusion assay. Somatostatin is a peptide hormone widely distributed in the body and plays an important role in regulating hormone secretion. Particularly, somatostatin release from the δ cells within pancreatic islets can effectively regulate pancreatic hormone release, including insulin. During the high (16.2 mM) glucose perifusion period (from minute 0 to 120), somatostatin (100 nM) was included transiently (from minute 60 to 90) in the buffer to test its inhibitory effect on the insulin release (Fig. 4*A*). Somatostatin suppressed insulin release in both wild-type and the heterozygous $G_{o2}\alpha^{+/-}$ islets. When somatostatin was removed from the buffer, insulin secretion resumed. Somatostatin suppressed insulin release from control and $G_{o2}\alpha^{+/-}$ islets to 59.2 ± 6.9% and 54.9 ± 2.2% of the rate observed after the somatostatin removal, respectively (Fig. 4*C*). However, in $G_{o2}\alpha^{-/-}$ islets, somatostatin did not inhibit insulin release, which only decreased to 95.0 ± 11.4% of the rate observed after the removal of somatostatin. The unchanged insulin secretion during and after somatostatin infusion demonstrates that somatostatin suppresses insulin secretion mainly through a G_{o2}-mediated mechanism.

The regulation of the insulin gene expression and the renewal of insulin stores are important aspects of normal homeostasis. Because $G_{o2}\alpha^{-/-}$ β cells secrete more insulin than wild-type controls, we looked at whether the expression of insulin genes is enhanced in $G_{o2}\alpha^{-/-}$ mice to accommodate the increased insulin secretory responses. Insulin gene expression was evaluated using quantitative, real-time reverse transcription PCR (qRT-PCR). As depicted in Fig. 4*D*, the expression of both the insulin I and the insulin II genes in $G_{o2}\alpha^{-/-}$ islets is comparable to that of wild-type controls. No difference in insulin gene expression was observed between $G_{o2}\alpha^{-/-}$ and wild-type islets, indicating that the enhanced insulin release from islets of $G_{o2}\alpha^{-/-}$ knockout mice results from a greater insulin secretory response to glucose. Next, we assessed the insulin content in $G_{o2}\alpha^{-/-}$ mice in both whole pancreas and isolated islets. The insulin content was 25.77 ± 2.19 and 17.77 ± 1.35 ng/mg pancreas for wild-type control and $G_{o2}\alpha^{-/-}$ mice, respectively. Thus, the insulin content in $G_{o2}\alpha^{-/-}$ mice was only 69% of that in controls, which is a significant reduction (P = 0.014) (Fig. 4*E*). We did not observe any obvious reduction in either the number or the size of islets in $G_{o2}\alpha^{-/-}$ mice compared with control in histological or anatomical analyses. We then determined the average insulin content in medium-sized islets. The average insulin per islet was 21.01 ± 0.54 ng for wild-type control islets and 14.27 ± 0.83 ng for $G_{o2}\alpha^{-/-}$ islets (Fig. 4*F*). Similar to observations in the whole pancreas, the insulin content is significantly reduced (P = 0.0076) in $G_{o2}\alpha^{-/-}$ islets, which contain only 70% of the insulin found in wild-type islets. This proportional reduction of insulin content in islets and pancreas suggests that the reduction of insulin content in $G_{o2}\alpha^{-/-}$ pancreas is the likely consequence of reduced insulin in islets, rather than a reduced number or size of the islets in $G_o2\alpha^{-/-}$ mice.

These results demonstrate that the absence of $G_{o2}\alpha$ results in insulin oversecretion and insulin content reduction, suggesting that G_{o2} mediates a critical negative regulatory pathway that prevents insulin oversecretion and preserves insulin content. Oversecretion of insulin into the bloodstream can lead to life-threatening hypoglycemia and increases the risk for developing insulin resistance. Hyperinsulinemia has been observed during the progression of type II diabetes. Prolonged exposure to high levels of circulating insulin leads to desensitization of peripheral tissues to insulin's action. During the progression of type II diabetes, reduced β-cell mass and insulin content are observed following hyperinsulinemia. When β-cell mass and insulin secretory capacity are no longer sufficient to compensate for insulin resistance, hyperglycemia appears. Normalization of plasma glucose levels by enhancing insulin output is a current therapeutic approach for treating type II diabetes. Despite treatments, β-cell failure occurs during the progression of disease and may result from depletion of insulin stores. Thus, the phenotypes of enhanced insulin secretion and reduced insulin content found in $G_o2\alpha^{-/-}$ mice model aspects of type II diabetes progression. However, the mechanism linking G_{o2} signaling to the preservation of insulin content is unclear. It is a logical assumption that G_{o2} mediates inhibition of insulin oversecretion and therefore maintains insulin stores.

Like the global $G_o\alpha^{-/-}$, islet-conditional $G_o\alpha$ total knockouts also yield a hyperinsulinemic phenotype (26). We show that the hyperinsulinemic phenotype is recapitulated by deletion of $G_{o2}\alpha$, but not by deletion of $G_{i1}\alpha$, $G_{i2}\alpha$, $G_{i3}\alpha$, or $G_{o1}\alpha$ in mice. Therefore, we conclude that deletion of G_{o2} in islets is the primary cause for the loss of inhibition of insulin release rather than the

secondary loss of $G_{o2}\alpha$ in other tissues. Complete blockade of all $G_{i/o}$ activity via β cell-specific expression of the PTX S1 subunit in mice has also been reported to induce hyperinsulinemia (39). Combined with our findings, this suggests that G_{o2} is the major target for PTX's insulin secretion stimulating (islet activating) properties. However, this study does not rule out the involvement of the other PTX-sensitive G proteins in regulating insulin secretion. Notably, after overnight fasting, β-cell PTX-expressing mice have a four- to fivefold increase in circulating insulin levels, whereas, $G_{o2}\alpha$-deficient mice have levels similar to wild-type despite having lower blood sugar levels. Loss of PTX-sensitive G protein activity, other than G_{o2}, may account for the rise of basal insulin secretion in PTX-expressing β cells. The finding of augmented basal insulin levels in PTX-expressing pancreatic islets and not in $G_{o2}\alpha$-deficient islets suggests a unique role for G_{o2} in tempering insulin secretion mainly after glucose stimulation. In addition, ablation of $G_z\alpha$, a PTX-insensitive member of the $G_{i/o}$ family, also results in augmented insulin secretion in mice (40). Loss of G_z caused an elevation of cAMP levels and resulted in ≈30% increase in insulin secretion after glucose challenge versus WT. The mice expressing PTX-S1 in β cells, which disrupts all $G_{i/o}$ activity but leaves the G_z-pathway unaffected, and $G_{o2}\alpha^{-/-}$ mice both exhibit a greater enhancement of glucose-induced insulin secretion than that seen in $G_z\alpha^{-/-}$ mice. This suggests that in islets the G_{o2}-mediated pathway is a more effective inhibitor of insulin secretion than the $G_z\alpha$-mediated pathway. Further study is required to determine whether the inhibitory effects of G_z and G_{o2} on insulin secretion occur through additive and/or independent mechanisms.

The α subunits of the PTX-sensitive subfamily of G proteins are highly homologous to each other (12). Although $G_{i/o}$ proteins can elicit similar signaling responses in cells, their functional overlap in the body remains to be elucidated. The divergence in expression pattern between G_i and G_o proteins alludes to unique functions for each. G_i proteins, expressed in most cells of the body, play a predominant role in antagonizing G_s activity by inhibiting adenylyl cyclase. Therefore, there is no surprise that a triple $G_{i1/2/3}\alpha$ knockout is early embryonic lethal. Individual $G_i\alpha$ knockouts and some combinations of double knockouts are viable and have normal growth rates, suggesting some degree of compensation and functional redundancy among G_i proteins. However, analysis of individual $G_i\alpha$ knockouts has yielded deficiencies unique to each $G_i\alpha$ subtype (15, 27, 41). The differing phenotypes suggest that there are preferential receptors and/or effectors coupling through each G_i protein. The G_o proteins, having a more restricted expression pattern, are found in neurons, endocrine cells, and at lower levels in other tissues, e.g., heart (42). $G_o\alpha$[total] knockout ($G_o\alpha^{-/-}$) animals have distinct phenotypes from the $G_i\alpha$ knockouts, which reflects poor functional compensation between G_i and G_o. Moreover, the level of functional redundancy between the two alternatively spliced $G_o\alpha$ isoforms has remained unclear. Using G_o isoform-specific knockouts, we have now identified unique and essential roles for these G_o isoforms. $G_o\alpha$[total] knockout animals exhibit reduced survival, enhanced insulin secretion, and neurological abnormalities and lack ERGs b-wave in retinal ON-bipolar cells (14, 29). Interestingly, the $G_{o1}\alpha$ and $G_{o2}\alpha$ isoform-specific knockout mice demonstrate mutually exclusive segregation of these phenotypes with reduced survival, neurological abnormalities, absent b-wave segregating with $G_{o1}\alpha^{-/-}$ mice (43), and abnormal insulin secretion and b-wave amplitude modulation segregating with $G_{o2}\alpha^{-/-}$ mice (44). This indicates that G_{o1} and G_{o2} govern distinct biological processes in the body. $G_{o1}\alpha$ and $G_{o2}\alpha$ share the same expression pattern; and, given their distinctive phenotypes in the knockouts, it is unlikely that heterotrimeric G_{o1} and G_{o2} are interchangeable for signaling transduction in the body. Because the 354-amino acid $G_o\alpha$ isoforms differ by only 25 of their 106 carboxyl-terminal residues (31), the segregation of $G_o\alpha[\text{total}]^{-/-}$'s phenotypes suggests a remarkably high level of G protein specificity. The C terminus of Gα subunits is critical for receptor binding and discrimination, suggesting that the G_{o1} and G_{o2} proteins may couple to different sets of receptors. In vitro studies suggest that PTX-sensitive $G_{i/o}$ G proteins could couple to similar types of receptors and elicit similar signaling responses (1, 20). However, gene ablation animals will be necessary to dissect functional differences among the subfamily and validate effector proteins reported in vitro.

Several G protein-coupled inhibitory hormone/neurotransmitter receptors have been identified in pancreatic islets, including those for somatostatin, galanin, ghrelin, and norepinephrine (1). Somatostatin, a peptide hormone secreted from δ cells within pancreatic islets, is released upon glucose stimulation. We observe an absence of somatostatin-induced inhibition on insulin secretion in $G_{o2}\alpha$-deficient islets, suggesting that G_{o2} is the primary transducer for somatostatin receptors in islet cells. Whether G_{o2} is the exclusive transducer for the other inhibitory hormones is unknown. It has been proposed that by potentiating K_{ATP} channel activity, inhibiting Ca^{2+} channels, and inhibiting adenylyl cyclase, inhibitory hormones can effectively suppress insulin release (1). Indeed, G_{o2} can mediate norepinephrine inhibition of insulin release by potentiating the K_{ATP} channels in an insulinoma cell line (45). Activation of the K_{ATP} channel, which hyperpolarizes the cell membrane, prevents insulin secretion from pancreatic islets, and modulation of the channel activity has been a therapeutic for treating type II diabetes. G_{o2} can also mediate somatostatin-induced inhibition of Ca^{2+} currents in the GH3 rat neuroendocrine cell line (46). Intracellular calcium is a central regulator for triggering the secretory machinery. Whether G_{o2} can inhibit adenylyl cyclase activity to suppress insulin secretion in β cells is under investigation. Furthermore, G_{o2} may directly regulate the insulin exocytosis process. G_o protein has been located on secretory granules in chromaffin cells (47) and may directly be involved in the exocytosis machinery (48). The constitutively activated $G_{o2}\alpha$ subunit itself can inhibit the insulin exocytosis process in an insulin-secreting cell line (49). The inhibitory hormones/neuropeptides, e.g., somatostatin (23), epinephrine (24), and galanin (25) can suppress the insulin exocytosis process in β-cell lines in a PTX-sensitive manner. We have observed that the readily releasable insulin pools are significantly reduced in conditional G_o knockout β cells (26). We show here that KCl-induced insulin release is also augmented in $G_{o2}\alpha^{-/-}$ β cells, supporting that the G_{o2}-mediated signaling may play a role in the exocytosis process. Interestingly, other members of $G_{i/o}$, $G_{i1/2}$, can mediate norepinephrine suppression of refilling of readily releasable pools of insulin in a cell line via their βγ subunits (50). The precise mechanisms remain to be determined.

Insulin secretion is necessary for the efficient transport of energy into tissues, and it is therefore tightly regulated to the availability of nutrients in the plasma. Negative regulation of insulin secretion serves to prevent hypoglycemia and to preserve insulin stores and peripheral tissue insulin sensitivity. This report is a unique physiological study of insulin secretion using specific $G_o\alpha$ isoform-deficient mice and shows that the G_{o2} isoform is an important negative regulator of insulin secretion. Of the $G_{i/o}\alpha$ knockout mice used in our study, only $G_{o2}\alpha$-deficient mice demonstrated significant improved glucose tolerance and hyperinsulinemia in the glucose-stimulated state. Furthermore, improved glucose handling in $G_{o2}\alpha$ knockouts is the direct result of augmented pancreatic insulin output rather than altered peripheral insulin sensitivity. In conclusion, our data indicate that G_{o2} is a major target of PTX and responsible for the islet activating effects of the toxin in β cells. $G_{o2}\alpha$ is indispensible for regulating normal glucose-stimulated insulin secretion from β cells because any redundant functions among other $G_{i/o}$ proteins cannot prevent oversecretion. The components in G protein signaling pathways, including G protein coupled receptors, and downstream effectors, such as enzymes and ion channels have been a rich source of targets for pharmaceuticals. The results presented here provide a possible direction for future investigations into interventions aimed at improving management of abnormal insulin secretion, both in the case of diabetes mellitus and persistent hyperinsulinemic syndromes.

Methods

Additional methods are described in *SI Methods*.

Generation of $G_{o1\alpha}$ and $G_{o2\alpha}$ Mutant Mice. $G_{o1}\alpha$ was inactivated by disrupting the exon 7.1 (Fig. S1). $G_{o2}\alpha$ was disrupted by introducing a stop codon in exon 7.2 (Fig. S2) using the two-step hit and run targeting strategy (36).

Measurements of Insulin Release in Islets. Pancreatic islets were isolated by collagenase digestion. A pool of islets was packed in a perifusion chamber for ex vivo insulin release assay in HKRB solutions and the insulin contents were determined by ELISA.

Insulin Content in Pancreas and Islets. Insulin in pancreata and islets was extracted with acid alcohol and the contents were determined by ELISA.

ACKNOWLEDGMENTS. We are especially indebted to Susanne Ullrich (Institute of Neurophysiology, University of Cologne) for her help in establishing the mouse pancreatic islet isolation technique in the laboratory. We thank MingHua Chai and Diana Vi for technical assistance. This work is supported by National Institutes of Health (NIH) Grants DK 069771 (to M.J.) and DK19319 (to L.B.) and in part by Intramural Research Program, NIH Project Z01- ES101643 (to L.B.).

1. Sharp GW (1996) Mechanisms of inhibition of insulin release. *Am J Physiol* 271: C1781–C1799.
2. Lang J (1999) Molecular mechanisms and regulation of insulin exocytosis as a paradigm of endocrine secretion. *Eur J Biochem* 259:3–17.
3. Ashcroft FM, et al. (1994) Stimulus-secretion coupling in pancreatic beta cells. *J Cell Biochem* 55(Suppl):54–65.
4. Zawalich WS, Zawalich KC (1996) Regulation of insulin secretion by phospholipase C. *Am J Physiol* 271:E409–E416.
5. Gulbenkian A, Schobert L, Nixon, Tabachnick II (1968) Metabolic effects of pertussis sensitization in mice and rats. *Endocrinology* 83:885–892.
6. Yajima M, et al. (1978) Islets-activating protein (IAP) in Bordetella pertussis that potentiates insulin secretory responses of rats. Purification and characterization. *J Biochem* 83:295–303.
7. Katada T, Ui M (1979) Islet-activating protein. Enhanced insulin secretion and cyclic AMP accumulation in pancreatic islets due to activation of native calcium ionophores. *J Biol Chem* 254:469–479.
8. Porte D, Jr. (1967) A receptor mechanism for the inhibition of insulin release by epinephrine in man. *J Clin Invest* 46:86–94.
9. Schnuerer EM, McDonald TJ, Dupre J (1987) Inhibition of insulin release by galanin and gastrin-releasing peptide in the anaesthetized rat. *Regul Pept* 18:307–320.
10. Reimer MK, Pacini G, Ahrén B (2003) Dose-dependent inhibition by ghrelin of insulin secretion in the mouse. *Endocrinology* 144:916–921.
11. Katada T, Ui M (1982) ADP ribosylation of the specific membrane protein of C6 cells by islet-activating protein associated with modification of adenylate cyclase activity. *J Biol Chem* 257:7210–7216.
12. Jiang M, Bajpayee NS (2009) Molecular mechanisms of Go signaling. *Neurosignals* 17: 23–41.
13. Jiang M, et al. (1998) Multiple neurological abnormalities in mice deficient in the G protein Go. *Proc Natl Acad Sci USA* 95:3269–3274.
14. Jiang M, Spicher K, Boulay G, Wang Y, Birnbaumer L (2001) Most central nervous system D2 dopamine receptors are coupled to their effectors by Go. *Proc Natl Acad Sci USA* 98:3577–3582.
15. Rudolph U, et al. (1995) Ulcerative colitis and adenocarcinoma of the colon in G alpha i2-deficient mice. *Nat Genet* 10:143–150.
16. Neer EJ, Lok JM, Wolf LG (1984) Purification and properties of the inhibitory guanine nucleotide regulatory unit of brain adenylate cyclase. *J Biol Chem* 259: 14222–14229.
17. Sternweis PC, Robishaw JD (1984) Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain. *J Biol Chem* 259: 13806–13813.
18. Homburger V, et al. (1987) Immunological localization of the GTP-binding protein Go in different tissues of vertebrates and invertebrates. *Mol Pharmacol* 31:313–319.
19. Worley PF, Baraban JM, Van Dop C, Neer EJ, Snyder SH (1986) Go, a guanine nucleotide-binding protein: immunohistochemical localization in rat brain resembles distribution of second messenger systems. *Proc Natl Acad Sci USA* 83:4561–4565.
20. Winzell MS, Ahrén B (2007) G-protein-coupled receptors and islet function-implications for treatment of type 2 diabetes. *Pharmacol Ther* 116:437–448.
21. Katada T, Ui M (1981) Islet-activating protein. A modifier of receptor-mediated regulation of rat islet adenylate cyclase. *J Biol Chem* 256:8310–8317.
22. Taussig R, Iñiguez-Lluhi JA, Gilman AG (1993) Inhibition of adenylyl cyclase by Gi alpha. *Science* 261:218–221.
23. Ullrich S, Prentki M, Wollheim CB (1990) Somatostatin inhibition of Ca2(+)-induced insulin secretion in permeabilized HIT-T15 cells. *Biochem J* 270:273–276.
24. Ullrich S, Wollheim CB (1988) GTP-dependent inhibition of insulin secretion by epinephrine in permeabilized RINm5F cells. Lack of correlation between insulin secretion and cyclic AMP levels. *J Biol Chem* 263:8615–8620.
25. Ullrich S, Wollheim CB (1989) Galanin inhibits insulin secretion by direct interference with exocytosis. *FEBS Lett* 247:401–404.
26. Zhao A, et al. (2010) Gαo represses insulin secretion by reducing vesicular docking in pancreatic beta-cells. *Diabetes* 59:2522–2529.
27. Pineda VV, et al. (2004) Removal of G(ialpha1) constraints on adenylyl cyclase in the hippocampus enhances LTP and impairs memory formation. *Neuron* 41: 153–163.
28. Toyota T, et al. (1978) Islet activating protein (IAP) derived from the culture supernatant fluid of Bordetella pertussis: Effect on spontaneous diabetic rats. *Diabetologia* 14:319–323.
29. Dhingra A, et al. (2000) The light response of ON bipolar neurons requires G[alpha]o. *J Neurosci* 20:9053–9058.
30. Bertrand P, Sanford J, Rudolph U, Codina J, Birnbaumer L (1990) At least three alternatively spliced mRNAs encoding two alpha subunits of the Go GTP-binding protein can be expressed in a single tissue. *J Biol Chem* 265:18576–18580.
31. Hsu WH, et al. (1990) Molecular cloning of a novel splice variant of the alpha subunit of the mammalian Go protein. *J Biol Chem* 265:11220–11226.
32. Strathmann M, Wilkie TM, Simon MI (1990) Alternative splicing produces transcripts encoding two forms of the alpha subunit of GTP-binding protein Go. *Proc Natl Acad Sci USA* 87:6477–6481.
33. Conklin BR, Farfel Z, Lustig KD, Julius D, Bourne HR (1993) Substitution of three amino acids switches receptor specificity of Gq alpha to that of Gi alpha. *Nature* 363: 274–276.
34. Hamm HE, et al. (1988) Site of G protein binding to rhodopsin mapped with synthetic peptides from the alpha subunit. *Science* 241:832–835.
35. Kleuss C, et al. (1991) Assignment of G-protein subtypes to specific receptors inducing inhibition of calcium currents. *Nature* 353:43–48.
36. Hasty P, Ramírez-Solis R, Krumlauf R, Bradley A (1991) Introduction of a subtle mutation into the Hox-2.6 locus in embryonic stem cells. *Nature* 350:243–246.
37. Jiang M, et al. (2002) Mouse gene knockout and knockin strategies in application to alpha subunits of Gi/Go family of G proteins. *Methods Enzymol* 344:277–298.
38. Moxham CM, Malbon CC (1996) Insulin action impaired by deficiency of the G-protein subunit G ialpha2. *Nature* 379:840–844.
39. Regard JB, et al. (2007) Probing cell type-specific functions of Gi in vivo identifies GPCR regulators of insulin secretion. *J Clin Invest* 117:4034–4043.
40. Kimple ME, et al. (2008) Galphaz negatively regulates insulin secretion and glucose clearance. *J Biol Chem* 283:4560–4567.
41. Gohla A, et al. (2007) An obligatory requirement for the heterotrimeric G protein Gi3 in the antiautophagic action of insulin in the liver. *Proc Natl Acad Sci USA* 104: 3003–3008.
42. Boknik P, et al. (2009) Genetic disruption of G proteins, G(i2)alpha or G(o)alpha, does not abolish inotropic and chronotropic effects of stimulating muscarinic cholinoceptors in atrium. *Br J Pharmacol* 158:1557–1564.
43. Dhingra A, et al. (2002) Light response of retinal ON bipolar cells requires a specific splice variant of Galpha(o). *J Neurosci* 22:4878–4884.
44. Okawa H, Pahlberg J, Rieke F, Birnbaumer L, Sampath AP (2010) Coordinated control of sensitivity by two splice variants of Gα(o) in retinal ON bipolar cells. *J Gen Physiol* 136:443–454.
45. Zhao Y, Fang Q, Straub SG, Sharp GW (2008) Both G i and G o heterotrimeric G proteins are required to exert the full effect of norepinephrine on the beta-cell K ATP channel. *J Biol Chem* 283:5306–5316.
46. Degtiar VE, Harhammer R, Nürnberg B (1997) Receptors couple to L-type calcium channels via distinct Go proteins in rat neuroendocrine cell lines. *J Physiol* 502: 321–333.
47. Toutant M, Aunis D, Bockaert J, Homburger V, Rouot B (1987) Presence of three pertussis toxin substrates and Go alpha immunoreactivity in both plasma and granule membranes of chromaffin cells. *FEBS Lett* 215:339–344.
48. Ohara-Imaizumi M, et al. (1992) Regulatory role of the GTP-binding protein, G(o), in the mechanism of exocytosis in adrenal chromaffin cells. *J Neurochem* 58:2275–2284.
49. Lang J, et al. (1995) Direct control of exocytosis by receptor-mediated activation of the heterotrimeric GTPases Gi and G(o) or by the expression of their active G alpha subunits. *EMBO J* 14:3635–3644.
50. Zhao Y, Fang Q, Straub SG, Lindau M, Sharp GW (2010) Noradrenaline inhibits exocytosis via the G protein βγ subunit and refilling of the readily releasable granule pool via the α(i1/2) subunit. *J Physiol* 588:3485–3498.