# Intra-islet insulin suppresses glucagon release via GABA-$GABA_A$ receptor system

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## Summary

**Excessive secretion of glucagon is a major contributor to the development of diabetic hyperglycemia. Secretion of glucagon is regulated by various nutrients, with glucose being a primary determinant of the rate of α cell glucagon secretion. The intra-islet action of insulin is essential to exert the effect of glucose on the α cells since, in the absence of insulin, glucose is not able to suppress glucagon release in vivo. However, the precise mechanism by which insulin suppresses glucagon secretion from α cells is unknown. In this study, we show that insulin induces activation of $GABA_A$ receptors in the α cells by receptor translocation via an Akt kinase-dependent pathway. This leads to membrane hyperpolarization in the α cells and, ultimately, suppression of glucagon secretion. We propose that defects in this pathway(s) contribute to diabetic hyperglycemia.**

## Introduction

Glucose stimulates insulin secretion from islet β cells but suppresses glucagon secretion from the α cells. A fine balance between insulin and glucagon secretion maintains the blood glucose levels within a narrow physiological range. However, the way these hormones are regulated within the pancreas is largely unknown.

Studies on islet microvasculature have shown that the α cells lie downstream from the β cells. Before insulin enters the circulation, its local intra-islet concentration is relatively high (Bonner-Weir and Orci, 1982). Insulin inhibits glucagon release within islets (Maruyama et al., 1984), likely via its action directly on the α cells (Kaneko et al., 1999; Kisanuki et al., 1995). In drug-induced insulin deficiency in dogs, glucose failed to suppress glucagon secretion in the absence of exogenous insulin (Greenbaum et al., 1991; Starke et al., 1987). These results support the concept that intra-islet insulin action is essential for suppression of glucagon in response to hyperglycemia (Muller et al., 1971).

In patients with type 2 diabetes, an oral glucose load induced a paradoxical rise in glucagon secretion, which could be normalized with optimal administration of insulin, suggesting that the dysfunctional regulation of pancreatic α cells in diabetes is related to insulin deficiency and an anomalous internal environment of the islets (Greenbaum et al., 1991; Hamaguchi et al., 1991). However, this defect is undefined because of an inadequate understanding of the mechanisms underlying suppression of glucagon by insulin in response to hyperglycemia.

Secretion of glucagon from α cells is regulated by various factors, including glucose, zinc, and the chemical transmitter γ-aminobutyric acid (GABA) (Pipeleers et al., 1985; Ishihara et al., 2003). The role of GABA and the A type GABA receptor ($GABA_AR$) in the regulation of glucagon release has been demonstrated (Braun et al., 2004; Rorsman et al., 1989). GABA is a major inhibitory neurotransmitter in the mammalian central nervous system (CNS) and is synthesized from glutamic acid by glutamic acid decarboxylase (GAD) (Gerber, 1980). Activation of $GABA_AR$, a $Cl^-$ ion channel, results in membrane hyperpolarization as a consequence of an inward $Cl^-$ flux (Kittler and Moss, 2003). In the CNS, $GABA_ARs$ are subject to modulation by their subunit composition, localization, number and phosphorylation states, and variance of GABA concentration in the synaptic cleft (Chebib and Johnston, 1999; Mody and Pearce, 2004). Pancreatic β cells contain high concentrations of GABA and GAD (Taniguchi et al., 1979). GABA is localized in "synaptic"-like microvesicles within β cells that are distinct from the insulin-containing large dense core vesicles, suggesting that exocytosis of pancreatic GABA is similar to the process found in neurons (Reetz et al., 1991; Sorenson et al., 1991). Functional $GABA_ARs$ are expressed in the α cells (Hales and Tyndale, 1994; Rorsman et al., 1989).

It has been proposed that, during hyperglycemia, GABA is co-released with insulin from the β cells and acts on $GABA_ARs$ on the α cells to reduce their secretion of glucagon (Rorsman et al., 1989). However, this hypothesis has been contested because of a lack of evidence for a parallel enhancement of GABA release with insulin in response to glucose stimulation (Rorsman et al., 1989). The failure to detect an increase in GABA release does not exclude the possibility that there is an increase in the responsiveness of $GABA_ARs$ on α cells upon hyperglycemia; however a clear-cut mechanism has not been delineated.

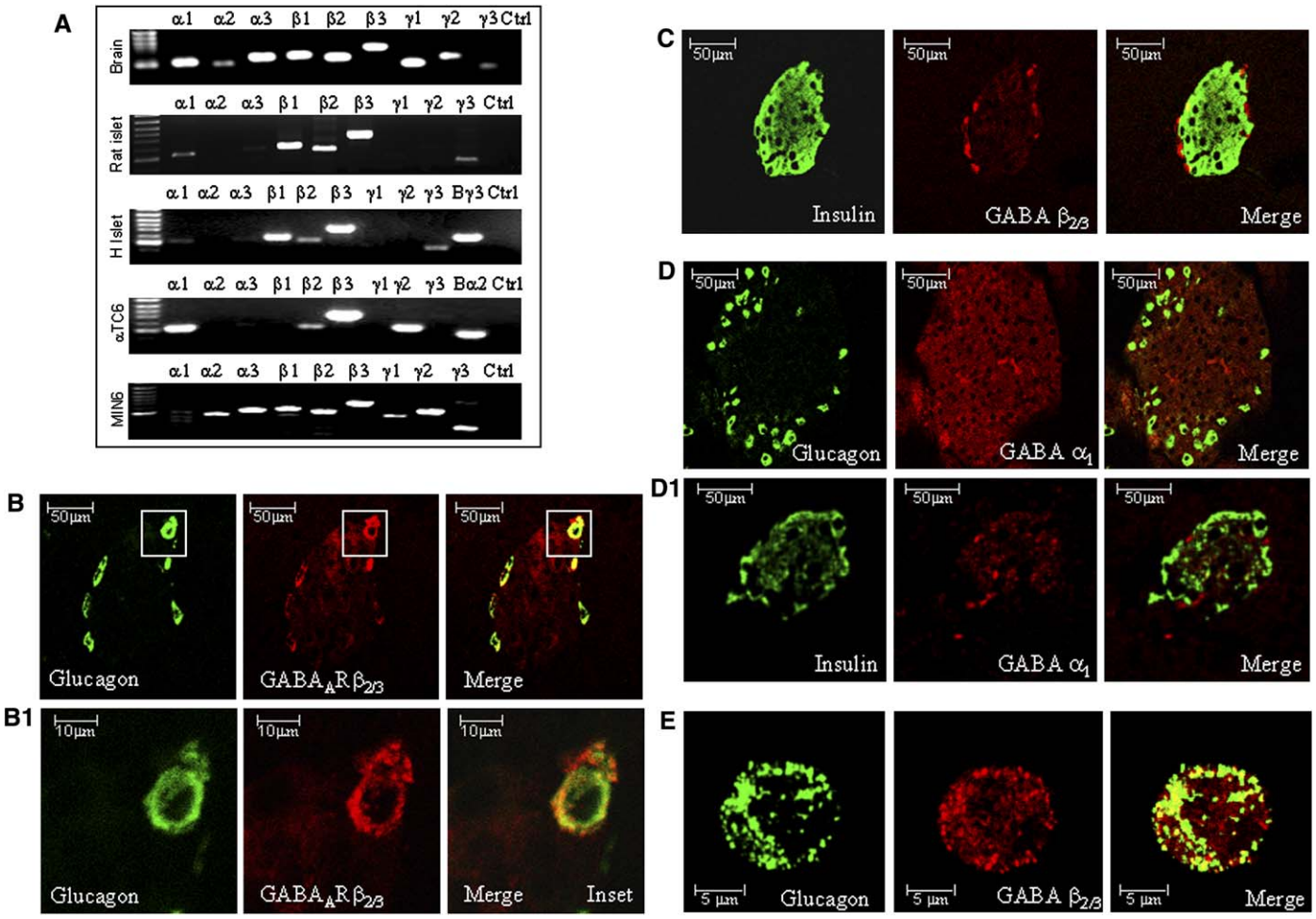


**Figure 1.** Expression of GABA$_A$R subunits in cell lines and islets

**A)** α (α-TC6) and β (MIN6) cell line and islet (human and rat) *mRNA* were used in one-step RT-PCR reactions using subunit-specific primers. Brain *mRNA* was used as a positive control while reactions devoid of *mRNA* were used as a negative control (Brain (B) $\gamma_3$, B$\alpha_2$).

**B)** Expression of β subunits within islet α cells. Representative confocal micrographs of pancreatic sections of mouse or rat pancreas (not shown) dual-stained for glucagon (green) and the GABA$_A$R ($\beta_{2/3}$ subunit, in red). The panels (**B1**) below represent high magnification of inset from (**B**).

**C)** Expression of the GABA$_A$R $\beta_{2/3}$ subunit (red) in mouse pancreatic β cells (stained in green with anti-insulin).

**D)** The α1 subunit appears to be ubiquitously colocalized with both glucagon (α cells, **D**) and insulin (β cells, **D1**).

**E)** Representative confocal micrographs of dispersed single rat islet cell stained for glucagon (green) and costained for the GABA$_A$R $\beta_{2/3}$ (red) subunit (n = 5, at least 100 cells were examined for each experiment).

While searching for an endogenous modulator regulating GABA$_A$R activity in pancreatic islets, we found that in cultured neurons and cells expressing functional GABA$_A$Rs, insulin can enhance GABA$_A$R activity via translocation of the receptors from an intracellular pool to the cell surface (Wang et al., 2003). In particular, Akt, a serine/threonine kinase, was identified as the key phosphorylating enzyme involved in conveying insulin signal to the GABA$_A$Rs (Wang et al., 2003). We now propose a mechanism whereby hyperglycemia-induced suppression of glucagon secretion from the α cells is through membrane translocation of GABA$_A$Rs via insulin action directly on the α cells. In this model, insulin rapidly activates Akt kinase, leading to phosphorylation and translocation of GABA$_A$R to the cell surface, thereby enhancing the responsiveness to GABA released from the β cells. Activation of the GABA$_A$Rs results in membrane hyperpolarization and suppression of glucagon secretion. We further propose that the excessive secretion of glucagon in diabetic subjects is a consequence of the ''insulin resistance'' that occurs in the α cells. As a result, the inhibitory effect of glucose on glucagon secretion is impaired because of defects in the insulin-Akt-GABA$_A$R-glucagon secretion pathway.

## Results

### Expression of GABA$_A$ receptor subunits in the α cells

The major subunit combination for a functional heteropentameric GABA$_A$R in the CNS has an αβγ configuration (Mody and Pearce, 2004). Using RT-PCR, we detected transcripts of α (mainly $\alpha_1$), $\beta_{(1-3)}$, and γ (mainly $\gamma_3$) GABA$_A$R subunits in both isolated rat and human pancreatic islet cells (Figure 1A). Similar results were obtained from glucagon-secreting mouse α-TC6 (Figure 1A) and IN-R1-G9 cells (data not shown). Variations in expression of individual GABA$_A$R subunits were noticed in α-TC6 cells with significant expression of γ2 rather than γ3 subunits and expression of lower levels of the β1 subunit (Figure 1A).

Northern blot analysis (not shown) further confirmed the expression of $GABA_AR$ subunit isoform transcripts $\alpha$ (mainly $\alpha_1$), $\beta$ ($\beta_{1\text{-}3}$), and $\gamma$ (mainly $\gamma_3$) in both isolated rat islets and $\alpha$ cell lines. The subunit expression and presumptive $GABA_AR$ configuration is in good agreement with previous reports (Rorsman et al., 1989; Wendt et al., 2004).

Immunostaining in paraffin-embedded mouse pancreatic sections revealed that $GABA_AR$ $\beta_{2/3}$ subunits were localized primarily within the glucagon-positive $\alpha$ cells (Figure 1B), while little was localized within the insulin-containing $\beta$ cells (Figure 1C). Pronounced expression of $GABA_AR$ in the $\alpha$ cells was also observed in rat pancreatic sections (data not shown). The staining pattern for the $\alpha_1$ $GABA_AR$ subunit was more diffuse and not as specific to one particular cell type within the pancreatic islets (Figure 1D). Indeed, RT-PCR (Figure 1A) and Northern blot (not shown) results showed that the $\alpha_1$ isoform (but not $\alpha_2$ or $\alpha_3$) is expressed within the rat and human islets, as well as the clonal cell lines (Figure 1A). Dispersed single-rat islet cells stained for glucagon and $GABA_AR$ $\beta_{2/3}$ subunits demonstrated that the $GABA_ARs$ were localized to the plasma membrane in addition to an intracellular reserve (Figure 1E). These findings indicate that pancreatic $\alpha$ cells express different $GABA_AR$ subunits, likely in a pattern whereby the $\alpha_1$ subunit combines with $\beta_{1/2/3}$ and $\gamma_3$ subunits.

### Insulin-induced $GABA_A$ receptor activation in $\alpha$ cells

To study the action of insulin on glucagon suppression in $\alpha$ cells, two well-characterized $\alpha$ cell lines were used, glucagon-secreting IN-R1-G9 (Drucker et al., 1991) and $\alpha$-TC6 cells (Yamada et al., 2001).

Immunostaining revealed a rapid translocation of Akt from the cytosol to the cell surface upon insulin stimulation in IN-R1-G9 cells (Figure 2A). Akt activation by insulin was congruent with translocation of the $GABA_AR$ to the plasma membrane (Figure 2B). A staining profile characteristic of membrane receptor clusters was observed in insulin-treated $\alpha$ cells (see arrows, Figure 2B), suggesting increased expression of functional receptors at the cell surface in response to insulin stimulation. These results were further confirmed in $\alpha$-TC6 cells (Figure 2C). Double labeling for the intracellular reserve pool (green) and plasma membrane-localized $GABA_ARs$ (red) showed an increase in the $GABA_ARs$ at the cell surface following insulin treatment, with reductions in intracellular reserves (Figure 2C), indicating that insulin stimulates $GABA_AR$ membrane translocation in $\alpha$ cells.

The role for Akt in mediating insulin action on $GABA_AR$ translocation was investigated using quantitative Cell-ELISA. In both $\alpha$-TC6 and IN-R1-G9 cells, insulin stimulation resulted in a dramatic increase in the density of $GABA_ARs$ at the cell surface (Figure 2D, $p < 0.01$). Notably, wortmannin (WM), an inhibitor of PI3-kinase, the Akt upstream molecule, diminished insulin-induced $GABA_AR$ translocation (Figure 2D). In contrast, inhibitors of PKA or PKC had no significant effect on insulin-induced $GABA_AR$ translocation (Figure 2D), excluding the involvement of these kinases in insulin-induced $GABA_AR$ translocation. These data indicate that insulin-stimulated $GABA_AR$ translocation occurs via a PI3-kinase/Akt-dependent pathway.

Insulin-induced $GABA_AR$ membrane translocation was also evident in rat islet $\alpha$ cells. Dual staining for $GABA_AR$ and glucagon or insulin demonstrated that $GABA_ARs$ were predominantly localized in the glucagon-containing cells (Figure 2E, "Ctrl") and very little in insulin-positive cells (Figure S1 available with this article online). Remarkably, the insulin-treated islet cells exhibited increased $GABA_ARs$ at the cell surface of the glucagon-containing cells (Figure 2E); this was blocked by WM pretreatment (Figure 2E) although WM, by itself, had no effect on the basal $GABA_ARs$ cellular distribution in the glucagon-positive islet cells ($p > 0.05$). The insulin-induced $GABA_ARs$ translocation is further illustrated in a representative line profile of fluorescent intensities across the glucagon-positive islet cells (Figure 2F, upper). Quantitative analysis of the ratio of membrane/cytosol (Figure 2F, middle) or membrane/total $GABA_AR$ (Figure 2F, lower) from at least 100 cells further indicated that insulin treatment resulted in significant receptor membrane recruitment ($p < 0.001$), which was significantly inhibited by WM ($\#p < 0.05$).

We have previously demonstrated, in neurons and HEK293 cells expressing $GABA_AR$, that Akt phosphorylates the $\beta_2$ subunit at serine 410 ($S_{410}$) within the major intracellular loop between TM3 and TM4 domains containing the RXRRRXS motif, a putative consensus Akt phosphorylation site (Wang et al., 2003). We thus determined if the RXRRRXS motif of the $\beta_1$, $\beta_2$, and $\beta_3$ subunits identified in $\alpha$ cells (Figure 2G) is also necessary for the $GABA_AR$ translocation. In vitro phosphorylation assays using glutathione S-transferase (GST) fusion proteins of the major intracellular loops of the $\beta_1$, $\beta_2$, or $\beta_3$ subunits of $GABA_AR$ showed that Akt kinase phosphorylated GST-$\beta_1$, GST-$\beta_2$, and GST-$\beta_3$, but not GST alone, or GST-$\alpha$ or $\gamma$ fusion proteins (Figure 2H). Notably, single-site mutants GST-$\beta_{1(S409A)}$, GST-$\beta_{2(S410A)}$, GST-$\beta_{3(S409A)}$ (but not GST-$\beta_{3(S408A)}$) completely abolished the Akt-mediated phosphorylation. For further validation, $GABA_AR$ translocation was determined in IN-R1-G9 cells transiently transfected with full-length cDNAs encoding $\alpha_1$, $\beta_1^{FLAG}$ (or $\beta_2^{FLAG}$, $\beta_3^{FLAG}$), and $\gamma_3$ subunits and stained for $GABA_ARs$ at the cell surface using anti-FLAG antibody. As shown (Figure 2I), insulin increased the numbers of $GABA_ARs$ at the cell surface in IN-R1-G9 cells transiently expressing $\alpha_1\beta_1\gamma_3$, $\alpha_1\beta_2\gamma_3$, or $\alpha_1\beta_3\gamma_3$ subunits of $GABA_AR$; this was dramatically reduced in the cells expressing $\alpha_1(\beta_{1(S409A)})\gamma_3$, $\alpha_1(\beta_{2(S410A)})\gamma_3$, $\alpha_1(\beta_{3(S409A)})\gamma_3$, or $\alpha_1(\beta_{3(S408AS409A)})\gamma_3$ but not in the cells expressing $\alpha_1\beta_{3(S408A)}\gamma_3$. These findings suggest that insulin-stimulated translocation of the $GABA_ARs$ to the cell surface on $\alpha$ cells occurs via phosphorylation of serine residues in the Akt consensus sites of the $\beta$ subunit receptor.

We next determined the effect of insulin on GABA-induced transmembrane current ($I_{GABA}$) using the perforated patch-clamp technique. In $\alpha$-TC6 cells, the GABA-evoked current was concentration dependent and sensitive to the $GABA_AR$ antagonist, bicuculline (BIC); the peak amplitude of $I_{GABA}$ in individual cells varied from 8 to 150 pA. Application of insulin enhanced $I_{GABA}$ by ~21% ($p < 0.01$, $n = 5$). This was prevented by WM treatment (Figures 3A and 3B, $p < 0.05$, $n = 5$), while the drug had no effects on $I_{GABA}$ under basal conditions (not shown). The effect of insulin on $I_{GABA}$ was also studied in isolated rat $\alpha$ cells. The $I_{GABA}$ was stable under control conditions and averaged 10.6 ± 1.6 pA; addition of insulin increased $I_{GABA}$ by 22% (Figures 3C and 3D, 1 ± 0.0 versus 1.22 ± 0.05, $p < 0.05$, $n = 3$). This effect was reversed by addition of WM (1.22 ± 0.05 versus 1.02 ± 0.10, $n = 2$). A similar effect of insulin and WM on $I_{GABA}$ was also observed in cells isolated from guinea pig islets (data not shown).

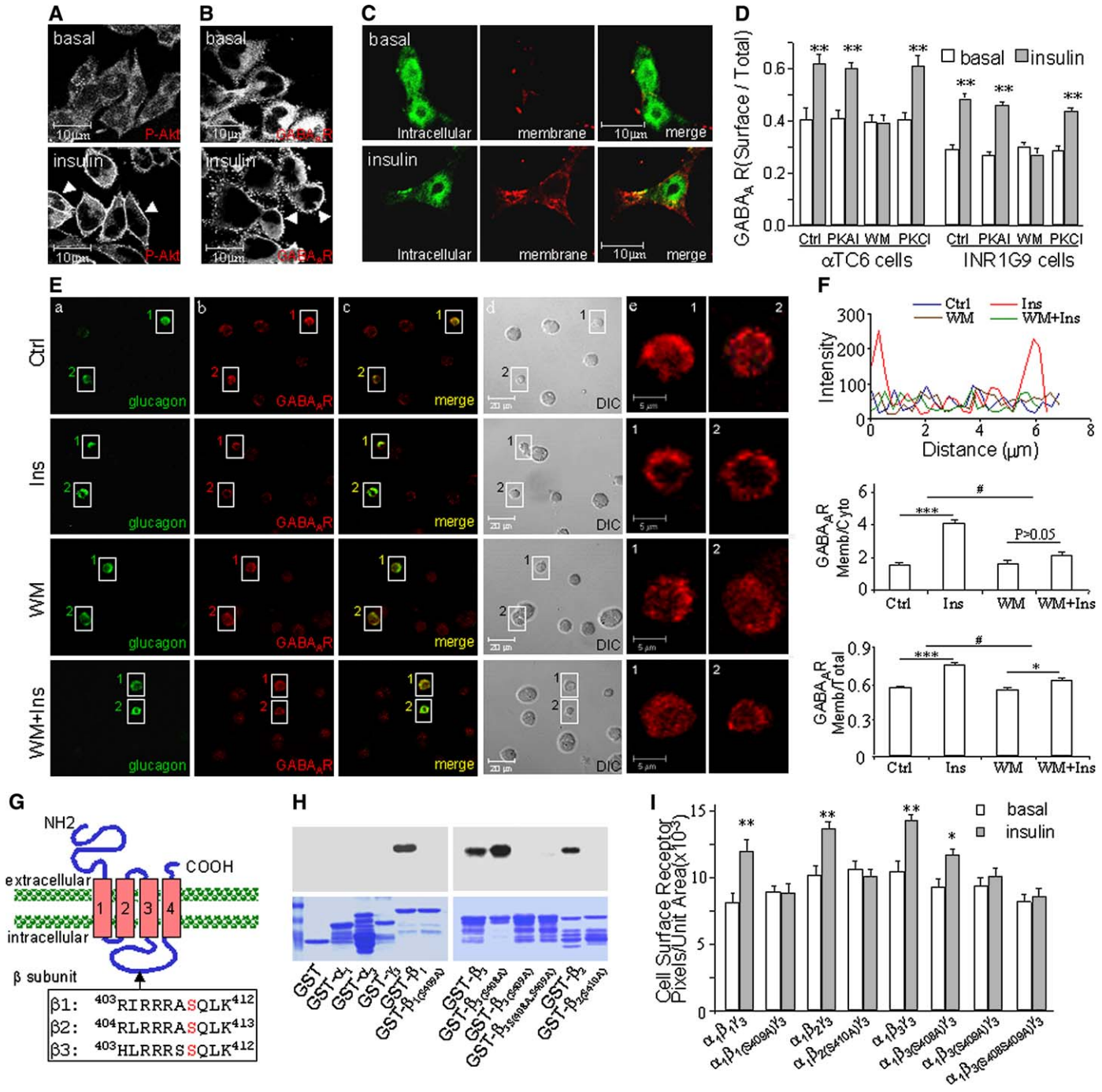


**Figure 2.** Insulin-induced GABA$_A$R plasma membrane translocation is Akt kinase dependent

**A–B)** Serum-starved IN-R1-G9 cells were treated with or without insulin (100 nM, 10 min) and immunostained for endogenous Akt (**A**) or GABA$_A$R ($\beta_{2/3}$ subunits) (**B**). Arrows indicate the translocation of Akt (**A**) and GABAAR (**B**) to the plasma membrane by insulin.

**C)** α-TC6 cells were treated as described above and were immunolabeled for the endogenous intracellular reserve pool of GABA$_A$Rs (green) and for the cell-surface-expressed receptors (red).

**D)** The cell-surface endogenous GABA$_A$Rs in α-TC6 and IN-R1-G9 cells were quantified by Cell-ELISA.

**E)** Insulin-induced GABA$_A$R membrane translocation is shown in dispersed rat islet cells. Cells with no treatment (Ctrl) or treated with 100 nM insulin (Ins, 10 min) were stained for glucagon (**a**, green), and endogenous GABA$_A$Rs $\beta_{2/3}$ subunit (**b**, red) and confocal images were taken as described in the Experimental procedures. The (**c**) panels are merged images of (**a**) and (**b**); the (**d**) panels are Differential Interference Contrast (DIC) images of the same field of views; images in the (**e1**) and (**e2**) panels are the glucagon-positive islet cells as framed in the left panels that are obtained under high magnification (×400). In parallel, some groups of cells were pretreated with 100 nM wortmannin (WM) for 15 min before being subjected to insulin treatment (WM+Ins). The data shown represent typical results of 3–6 independent experiments (at least 100 cells were examined for each experimental condition). GABA$_A$Rs were found to be localized primarily within the glucagon-positive cells (**E**).

**F)** Upper: A representative line profile of fluorescence intensities across a single glucagon-positive islet cell along a preset line for the distance increment is shown (Ctrl, no treatment; Ins, treatment of 100 nM insulin for 10 min; WM, treatment of 100 nM wortmannin; WM+Ins, treatment of 100 nM insulin for 10 min in the presence of WM). Quantification was made from at least 100 cells and expressed as a ratio of membrane/cytosol (**F**, middle) or membrane/total GABA$_A$R (**F**, lower).

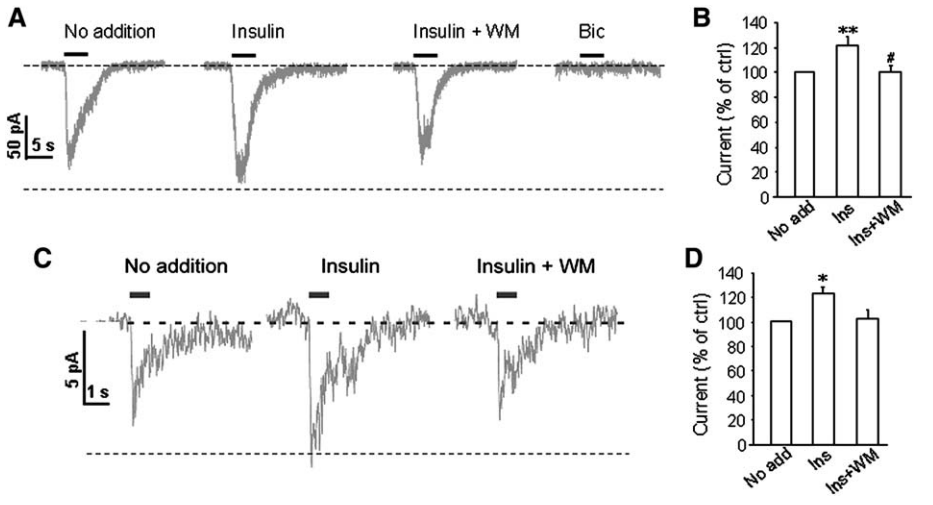


**Figure 3.** Insulin potentiates GABA evoked currents in $\alpha$ cells

**A)** Effect of insulin and wortmannin (WM) on GABA-induced current, $I_{GABA}$, in $\alpha$-TC6 cells was determined using the perforated patch whole-cell configuration. GABA (1 mM) evoked currents in $\alpha$-TC6 cells under conditions of control (no addition), insulin treatment (Ins, 100 nM, 8–10 min), pretreatment with wortmannin (100 nM, 10 min) plus insulin (Ins+WM), and pretreatment with bicuculline (Bic, 100 $\mu$M, 5 min).

**B)** The bar graph shows the normalized GABA currents (to "no addition") from five independent experiments (no add. versus Ins = 1 ± 0.0 versus 1.21 ± 0.05, *$p < 0.01$; Ins versus Ins+WM = 1.21 ± 0.06 versus 1.01 ± 0.05, #$p < 0.05$).

**C)** Current responses were elicited by application of GABA (1 mM) to rat $\alpha$ cells. Currents were measured in the perforated-patch configuration before (no addition) or 5 min after application of insulin (100 nM) or wortmannin (100 nM, 10 min) plus insulin (Ins+WM) to the bath.

**D)** Bar graph shows normalized GABA currents (to no addition) from three independent experiments except the WM data was obtained from two independent experiments. Data of $\alpha$-TC6 cells and rat islet $\alpha$ cells were obtained from the same cells and are presented as means ± SEM (no add. versus Ins = 1 ± 0.0 versus 1.22 ± 0.05, *$p < 0.05$; Ins versus Ins+WM = 1.22 ± 0.05 versus 1.02 ± 0.10). The significance was examined by one-way ANOVA test and Post Hoc Tukey (for $\alpha$-TC6 cells) or Student's t test (for rat islet $\alpha$ cells).

### Insulin-induced activation of GABA$_A$Rs leads to suppression of glucagon secretion

Based on the outlined hypothesis, we expect that blocking either insulin signaling or inactivating GABA$_A$Rs in $\alpha$ cells should block suppression of glucagon secretion from the islet $\alpha$ cells.

To verify, a "paracrine" GABA environment was simulated in $\alpha$ cells by adding 10 nM GABA, a physiologically appropriate GABA concentration (Winnock et al., 2002). We observed that, under these conditions, application of insulin suppressed glucagon secretion by ~35% in $\alpha$-TC6 cells ($p < 0.001$, Figure 4A). Whereas WM completely reversed insulin-suppressed glucagon secretion, the drug had little effect on basal glucagon secretion (Figure 4A). Inactivation of the GABA$_A$Rs by coapplication of BIC also significantly reversed the insulin action on glucagon secretion ($p < 0.05$), although BIC had no significant effects on basal glucagon secretion ($p > 0.05$, Figure 4A). Similar results were obtained with IN-R1-G9 cells (Figure 4B). These results indicate that suppression by insulin of glucagon secretion in the $\alpha$ cells was abolished when the insulin signaling was blocked or GABA$_A$Rs were inactivated in the $\alpha$ cells.

To examine the mechanism in a more physiological setting, we measured glucagon secretion in isolated rat islets where, under high-glucose conditions, GABA release from the $\beta$ cells is unchanged (Smismans et al., 1997; Rorsman et al., 1989), while insulin release is increased and can act on the $\alpha$ cells. As shown, glucose treatment resulted in a significant reduction of glucagon secretion (Figure 4C) and was reversed by WM or BIC, although the drugs by themselves had no significant effects on the glucagon release (Figure 4C).

Correspondingly, in human islets that express GABA$_A$Rs primarily in the glucagon-positive $\alpha$ cells (Figure 4D), glucose significantly enhanced insulin production (Figure 4F, $p < 0.01$) and reduced glucagon secretion (Figure 4E, $p < 0.01$). WM abolished the glucose-induced reduction in glucagon release (Figure 4E, $p < 0.05$), with no significant effect on glucose-stimulated insulin secretion from the islets (Figure 4F). Moreover, glucose-suppressed glucagon secretion was also reversed upon inactivation of the GABA$_A$Rs by BIC (Figure 4E, $p < 0.05$), although the drug had no significant effect on glucose-stimulated insulin secretion (Figure 4F). At low glucose (2 mM), glucagon secretion was not affected by WM or BIC (Figure 4E), but insulin secretion tended to increase (Figure 4F, $p > 0.05$). Although preliminary, these results support the notion that the suppressive effect of glucose on glucagon secretion is mediated by the action of insulin on the $\alpha$ cells via PI3-kinase/Akt-dependent modulation of GABA$_A$R activity.

To rule out extraneous pharmaceutical effects on intracellular protein kinases or channel activities in the $\alpha$ cells and to further confirm that insulin's action on glucagon secretion is mediated by Akt kinase and GABA$_A$Rs, we developed complementary experiments directly targeting Akt or the $\beta$ subunit(s) of GABA$_A$R. Viral transduction using adenoviral vector constructs encoding wild-type (Ad-wt), dominant-negative (Ad-DN), or constitutively active (Ad-CA) Akt (Katome et al., 2003) resulted in a dose-

**G)** A schematic representation of membrane topology of GABA$_A$R $\beta$ subunits and an alignment of the conserved putative Akt kinase target sequence in the intracellular loop between transmembrane domains 3 and 4 (targeted serine residue is highlighted in red).

**H)** GST-fusion proteins containing the intracellular loop between transmembrane domains 3 and 4 of $\alpha_1$, $\alpha_3$, $\gamma_3$, $\beta_1$, $\beta_{1(S410A)}$, $\beta_3$, $\beta_{3(S408A)}$, $\beta_{3(S409A)}$, $\beta_{3\ (S408A/S409A)}$, $\beta_2$, $\beta_{2(S410A)}$, along with GST alone as a control, were subjected to in vitro phosphorylation assays using recombinant active Akt1 (see Experimental procedures). The assay mixtures were subjected to SDS-PAGE and visualized by autoradiography (top). Coomassie blue staining of the same SDS gel shows that similar amounts of GST fusion proteins were used in each reaction (bottom).

**I)** The GABA$_A$R translocation was examined on images of the IN-R1-G9 cells transiently transfected with plasmids encoding GABA$_A$R $\alpha_1$, $\beta_1^{FLAG}$ (or $\beta_2^{FLAG}$, $\beta_3^{FLAG}$), and $\gamma_3$ subunits (DNA ratio = 2:2:1) or plasmids of GABA$_A$R subunits with the same combinations but the $\beta$ subunits were replaced by various mutants ($\beta_{1(S409A)}$, $\beta_{2(S410A)}$, $\beta_{3(S408A)}$, $\beta_{3(S409A)}$, or $\beta_{3(S408A/S409A)}$). Transfected cells were serum deprived, stimulated with insulin (100 nM, 10 min), and stained for the cell surface GABA$_A$Rs using anti-FLAG antibody under nonpermeant condition.

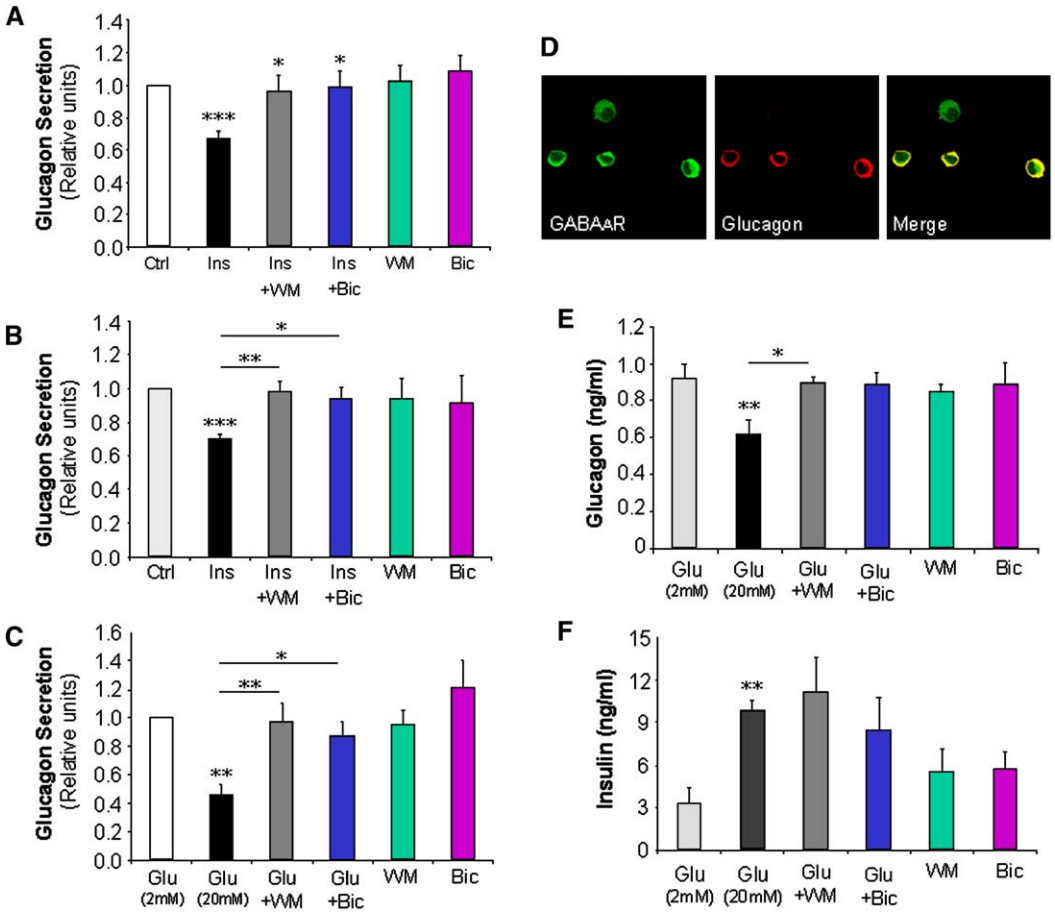


**Figure 4.** Insulin-suppressed glucagon secretion from α cells is wortmannin and/or bicuculline sensitive

Glucagon secretion from α-TC 6 cells (**A**), In-R1-G9 cells (**B**), isolated rat islets (**C**), and human islets (**E**) was measured in KRB using RIAs. Briefly, Serum-deprived cells (**A** and **B**) were incubated with 100 nM insulin alone (Ins), 100 nM insulin plus 100 nM WM (Ins+WM), or 100 nM insulin plus 10 μM BIC (Ins+Bic) or incubated with WM or BIC alone during the glucagon secretion assays (n = 6–12). GABA (10 nM) was used during the incubation period to mimic the paracrine environment (see Figure 7 and Experimental procedures). Glucagon secretion from rat (**C**) and human (**E**) islets was assayed as described above for (**A**) and (**B**) except that GABA was omitted and glucose was used instead of insulin (n = 13 [rat], n = 4 [human]). The "n" corresponds to the number of separate independent experiments performed with rats; with the human assays, "n" corresponds to the number of experiments that were performed repeatedly using islets from the same donor. Images (**D**) were obtained by confocal microscopy using dispersed human islet cells coimmunostained for $GABA_AR$ ($\beta_{2/3}$, green) and glucagon (red). Insulin secretion (**F**) from isolated human islets under experimental conditions as described in (**E**) was measured using RIA. *p < 0.05, **p < 0.01, ***p < 0.001.

dependent increase in the expression of the three types of Akt, while transduction of vector itself (Ad-*g*reen *f*luorescent *p*rotein [GFP]) had no noticeable effect on endogenous Akt expression (Figure 5A). Insulin-stimulated Akt phosphorylation (pS473) in the transduced cells was viral concentration dependent (Figure 5B). An Akt kinase assay (Figure 5C) showed that insulin increased Akt kinase activity in the nontransduced cells (~15-fold, $p < 0.001$) and the GFP-expressing cells (~14-fold, $p < 0.001$). Akt kinase activity under basal conditions was relatively high in cells expressing Ad-wt-Akt (~10-fold over basal, $p < 0.01$); insulin treatment further enhanced Akt activity by ~5-fold ($p < 0.01$). However, insulin had no further stimulatory effects on Akt activation in cells expressing Ad-CA-Akt, as the kinase activity was significantly elevated under basal conditions (~40-fold, $p < 0.005$) (Figure 5C). In contrast, in the cells expressing Ad-DN-Akt, while basal activity was not significantly altered, insulin-stimulated Akt activation was completely abolished (Figure 5C).

The transduction procedures had no effect on the glucagon secretion, as measured in parallel in virally transduced α cells (Figure 5D). In the presence of basal levels of GABA (10 nM), insulin suppressed glucagon secretion in cells expressing Ad-GFP by ~34% ($p < 0.001$) and in cells expressing Ad-wt-Akt by ~29% ($p < 0.005$). On the contrary, this suppressive effect was completely abolished in cells expressing Ad-DN-Akt ($p < 0.05$). In the absence of insulin, glucagon secretion was modestly but significantly suppressed in cells expressing Ad-wt-Akt (~15%, $p < 0.05$); further suppression was seen in cells expressing Ad-CA-Akt (~34%, $p < 0.001$). These findings indicate that ablating Akt kinase activity diminishes insulin-induced suppression of glucagon secretion. Moreover, as elevation of Akt kinase activity mimics insulin effects, activation of Akt kinase appears to be sufficient to suppress glucagon secretion in α cells.

### Insulin-suppressed glucagon secretion is diminished in a cellular model of insulin resistance

We further investigated insulin-directed $GABA_AR$ membrane trafficking and glucagon secretion in a cellular model of insulin resistance. This model was established by incubating In-R1-G9

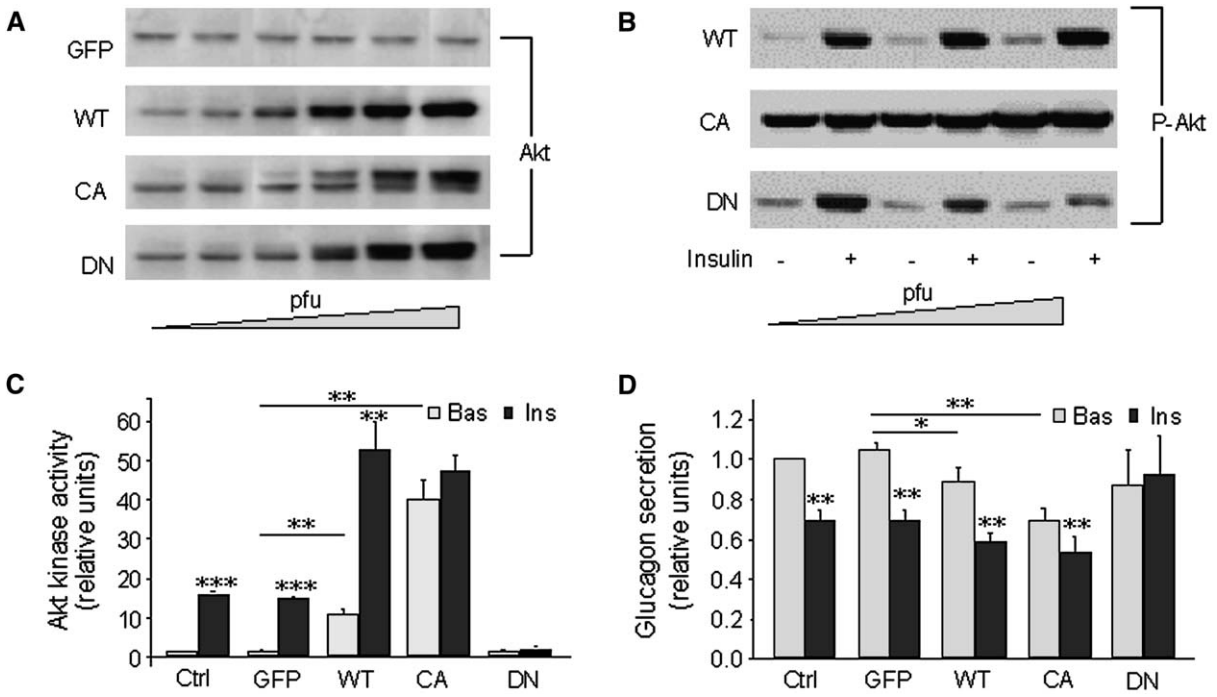


**Figure 5.** Insulin-suppressed glucagon release is mediated by the Akt-GABA$_A$R pathway

In-R1-G9 cells were transduced with various GFP-tagged adenovirus carrying Akts (GFP, green fluorescent protein; wt, wild-type Akt; CA, constitutively active Akt; DN, dominant-negative Akt). Serum-deprived cells were treated with ("+") or without ("−") insulin (100 nM, 5 min), and total Akt protein content (**A**) or Akt phosphorylation (**B**) was determined by Western blotting using anti-Akt or anti-phospho-Akt antibodies. A transduction rate >99% of IN-R1-G9 cells was obtained at a viral concentration of ~1 × $10^8$ pfu/ml as determined by GFP fluorescence.

**C)** Serum-deprived In-R1-G9 cells transduced with various viral constructs, or nontransduced control cells (Ctrl) were incubated with vehicle (blank bars) or insulin (100 nM, 5 min; black bars). Following the treatment, total Akt was immunopurified and kinase activity was determined using the specific Akt substrate Crosstide (see Experimental procedures).

**D)** Under similar transduction conditions, glucagon was measured in IN-R1-G9 cells transduced with various Ad-Akts using RIAs.

α cells in culture medium containing high concentrations of glucose (25 mM) and insulin (100 nM) (high Glu/Ins) for 24 hr (as previously described for muscle; [Huang et al., 2002], adipocytes [Lu et al., 2001], or liver cells [Nakajima et al., 2000]). We found that acute insulin stimulation stimulated tyrosine phosphorylation of the insulin receptor (IR) in the control cells ($p < 0.001$); this was significantly diminished after 24 hr of high Glu/Ins pretreatment (Figure 6A, $p < 0.01$). As expected, insulin dramatically stimulated Akt activation in the control cells as examined by Akt phosphorylation (~12-fold, Figure 6B, $p < 0.001$) and kinase assays (~14-fold, Figure 6C, $p < 0.001$). This was significantly reduced after a 24 hr preincubation with high Glu/Ins (Figure 6C, $p < 0.01$), although chronic high Glu/Ins had no significant effect on protein levels of IR (Figure 6A, $p > 0.05$) or Akt (Figure 6B, $p > 0.05$). These results suggest that, despite an elevation in basal Akt activity (~3-fold, Figures 6B and 6C, $p < 0.01$), a significant reduction of signal transduction following acute insulin stimulation occurred in these α cells after chronic high Glu/Ins treatment.

Under identical experimental conditions, we assayed glucagon secretion and found a significant suppression of glucagon secretion by insulin in the control cells (33%, $p < 0.01$). However, while the basal glucagon secretion was markedly decreased (Figure 6D, $p < 0.05$), acute insulin treatment did not further suppress glucagon release in cells chronically exposed to high Glu/Ins (Figure 6D, $p > 0.05$). These data suggested that insulin was not able to suppress glucagon secretion when insulin signaling was blunted. Of note, chronic treatment with Glu/Ins resulted in a relatively small, yet significant, elevation in Akt kinase activity (Figure 6C, $p < 0.01$; Glu/Ins versus Ctrl = ~3-fold versus ~14-fold). This "basal" elevation of Akt activity (Figure 6C) thus appeared to have a significant suppressive effect on glucagon secretion (Figure 6D, $p < 0.05$).

To determine whether this "basal" elevation of Akt activity is sufficient to cause translocation of GABA$_A$Rs and hence the subsequent suppression of glucagon release, we examined the distribution of GABA$_A$Rs in the α cells following chronic treatment with high Glu/Ins. Cell-surface GABA$_A$R staining and quantification revealed that, in control In-R1-G9 cells, insulin significantly increased GABA$_A$R membrane translocation (Figure 6E, upper-left, $p < 0.01$). However, acute insulin treatment did not significantly increase GABA$_A$R membrane translocation in cells pretreated with high Glu/Ins (Figure 6E, upper-right, $p > 0.05$) likely due to a significant basal elevation of the number of GABA$_A$R at the cell surface (Figure 6E, $p < 0.01$). These observations indicate that increased GABA$_A$R translocation associated with decreased glucagon production after chronic high Glu/Ins treatment is mostly due to the elevation of the basal Akt kinase activity. Therefore, GABA$_A$R translocation, in response to transient increases in insulin, is a prerequisite in modulating glucagon secretion.

## Discussion

Excessive glucagon secretion from pancreatic α cells is a major contributor to the development of diabetic hyperglycemia.

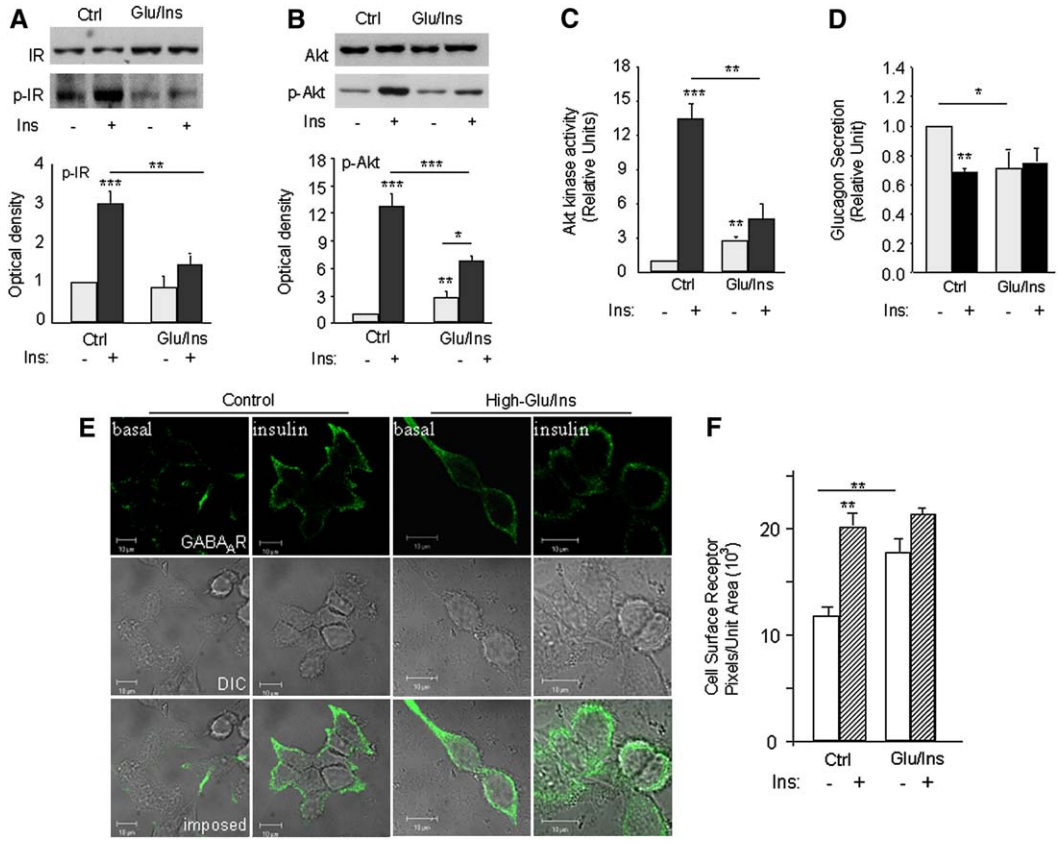


**Figure 6.** Exposure of IN-R1-G9 cells to high glucose and insulin reduces insulin signaling associated with diminished insulin suppression on glucagon secretion

**A–B)** IN-R1-G9 cells were incubated for 24 hr in control or high Glu/Ins medium (glucose 25 mM, insulin 100 nM). Total cell lysates (~50 μg) were subjected to Western blotting for (**A**) IR, phospho-IR, (**B**) Akt and phospho-Akt.

**C)** Total Akt proteins were immunopurified and the Akt kinase activity was determined.

**D)** Glucagon from the medium was assayed using RIA with control IN-R1-G9 cells or IN-R1-G9 cells treated with high Glu/Ins. Results are the means ± SEM of 5–6 experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

**E)** Translocation of GABA$_A$Rs in the control (left two panels) or the high-Glu/Ins-treated IN-R1-G9 cells (right two panels) was determined by cell-surface staining of endogenous GABA$_A$R ($\beta_{2/3}$). The representative images show that insulin-induced GABA$_A$R translocation occurs in the control cells (left, top panel) but not in the cells treated with high Glu/Ins. The middle row shows the Differential Interference Contrast (DIC) images of the same cells shown in the top panels; bottom row shows the merged images of DIC and fluorescent. (**F**) The bar graph shows the quantitative results of the cell-surface staining (means ± SEM of 100 to 300 cells analyzed).

Understanding the physiological mechanisms controlling glucagon secretion and the causes of hyperglucagonemia is therefore vital to understanding diabetes.

In this report, we demonstrate that glucose-induced suppression of glucagon release occurs by means of insulin-induced membrane translocation/activation of the GABA$_A$Rs in α cells (Figure 7). Our results indicate that the insulin-induced GABA$_A$R translocation/activation is Akt kinase dependent but not dependent on other kinases such as PKA or PKC. This is supported, at least in part, by evidence from previous studies. First, although both $\beta_1$, and $\beta_3$ subunits have been previously shown to be phosphorylated by both PKA and PKC, PKC-induced phosphorylation leads to a reduction of GABA$_A$R function (Brandon et al., 2000; McDonald and Moss, 1997). Second, PKC-induced phosphorylation of the neuronal $\beta_3$ subunit occurred on both S408 and S409 (Brandon et al., 2000). In contrast, Akt-mediated phosphorylation of the $\beta_3$ subunit occurs only on S409. Third, modulation of GABA$_A$R by PKC occurred mainly via changing channel gating or conductance rather than altering GABA$_A$R trafficking (Brandon et al., 2000). Finally, the PKA signaling is likely to be distinct from the signaling pathway(s) following IR activation, as IN-R1-G9 cells used in these studies have been found to be PKA deficient (Drucker et al., 1991). Therefore, insulin-induced GABA$_A$R membrane translocation/activation in the α cells appears to be solely dependent on Akt kinase activity.

Our data are in line with previous findings that blocking GABA$_A$Rs attenuated GABA-induced suppression of glucagon secretion in guinea pig islets (Rorsman et al., 1989) or rat islets (Wendt et al., 2004). Rodent α cells have been shown to possess a low resting membrane conductance (0.1–0.4 nS) (Barg et al., 2000; Bokvist et al., 1999). Under these conditions, a current a few pA in amplitude can substantially affect the membrane potential. It is therefore conceivable that a 22% increase of the GABA$_A$R-mediated current leads to a significant hyperpolarization of the cell and reduction of electrical activity. Our data further show that inactivating Akt kinase by using either pharmacological agents or adenoviral-delivered dominant-negative constructs dramatically abolished insulin-suppressed glucagon secretion, indicating that insulin-induced suppression of glucagon release requires Akt activity. Moreover, bypassing IR activation by elevating Akt kinase activity by adenoviral delivery of

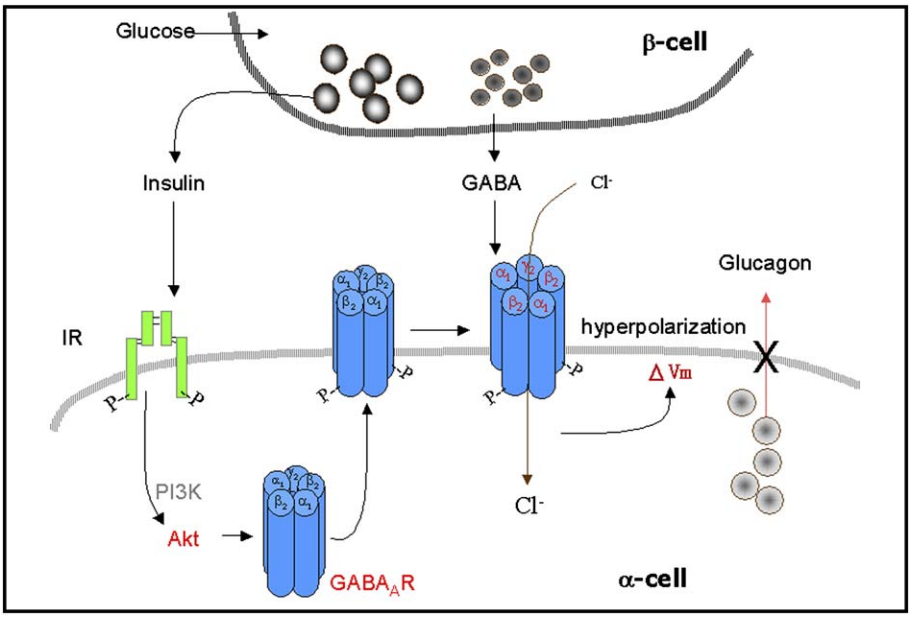


**Figure 7.** A model showing the Akt-GABA$_A$R pathway in mediating glucose-induced suppression of glucagon release in α cells

When glucose levels increase, insulin released from β cells activates the IR present on α cells. Activation of Akt leads to phosphorylation of β subunits of GABA$_A$R that causes rapid translocation of the receptor to the plasma membrane. As GABA is constantly released from β cells (Rorsman et al., 1989), increased GABA$_A$R numbers at the cell surface increases the efficacy of the receptor-mediated inhibitory currents ($Cl^-$) and membrane hyperpolarization occurs. In turn, membrane hyperpolarization shuts down voltage-dependent $Ca^{2+}$ channels (Göpel et al., 2004) and as a result inhibits α cell exocytosis and glucagon release. The $Ca^{2+}$-dependent exocytosis machinery is not shown.

CA-Akt significantly suppressed glucagon secretion, suggesting that activation of Akt kinase is sufficient to suppress glucagon release. In our present study, the Akt vectors encode Akt1 (Katome et al., 2003) and the antibody used in the Akt kinase assays recognizes only Akt1. We thus believe that Akt1 may be the predominant isoform that mediates insulin action on the α cells.

Interestingly, an earlier study (Gilon et al., 1991) showed that, in the presence of arginine, glucose-induced inhibition of glucagon release was not altered by bicuculline. The reasons for this discrepancy are unclear and further study is required. Nonetheless, although we believe that the receptor translocation is a major mechanism leading to an increase in GABA$_A$R number at the α cell surface upon insulin stimulation, we cannot rule out the possibility that insulin stimulation might increase the stability of the GABA$_A$Rs on the cell surface (Luscher and Keller, 2004) or lead to a reduction in endocytosis (Cinar and Barnes, 2001). Further experiments are required to clarify the mechanism.

Previous insulin infusion studies in diabetic animal models (Greenbaum et al., 1991; Starke et al., 1987) and diabetic patients (Hamaguchi et al., 1991) demonstrated that, while glucose alone could not suppress glucagon secretion, insulin suppressed glucagon release in the diabetic subjects. These in vivo studies indicated a crucial role for intra-islet insulin in permitting glucose to suppress glucagon secretion (Greenbaum et al., 1991; Starke et al., 1987). Our current findings provide new insights into the mechanisms underlying insulin-induced suppression of glucagon secretion at the cellular and molecular level.

In the α cells, while chronic treatment with high Glu/Ins significantly elevated basal Akt activity that was associated with increased GABA$_A$R numbers at the cell surface and suppressed glucagon secretion, the subsequent addition of insulin did not further increase GABA$_A$R numbers at the cell surface or further suppress glucagon secretion. Though these results indicate that elevation of Akt activity with consequent GABA$_A$R membrane translocation is sufficient to suppress glucagon secretion in the α cells, they do not fully explain the situation in diabetic subjects, i.e., diabetic hyperglucagonemia. Presumably these findings reflect the compensatory mechanism(s) of the islet cells (e.g., enhanced insulin secretion and reduced glucagon secretion to compensate for peripheral insulin resistance) in the early stage of insulin resistance (Bergman et al., 2002; Ferrannini, 1998). Yet, it is conceivable that when insulin resistance becomes severe, β cells may lose their capacity to compensate and diabetic hyperglucagonemia might develop as a result of attenuated insulin action on the α cells. Further experiments using diabetic animal models are required to address this possibility.

An impaired counter-regulatory response to hypoglycemia is a critical problem for diabetic patients receiving exogenous insulin treatment (Cryer, 1994). Studies in humans demonstrated that intra-islet hyperinsulinemia is the major contributor that prevents the glucagon response to hypoglycemia (Banarer et al., 2002). The importance of insulin in modulating the α cell secretion has been highlighted recently by the "switch-off" hypothesis that the proper responsiveness of α cells to hypoglycemia requires "turning off" insulin action on the α cells (Hope et al., 2004; Zhou et al., 2004). The question raised by our present results is whether the artificially high levels of insulin given to diabetic patients might chronically upregulate GABA$_A$R in the α cells, turning off the ability to secrete glucagon in insulin-induced hypoglycemia. This is a testable hypothesis and will be explored in the future.

Although activation of the insulin-Akt-GABA$_A$R pathway under high-glucose conditions appears to be a prominent mechanism underlying glucose-suppressed glucagon secretion in α cells, this pathway may not be the sole mechanism regulating glucagon secretion. For example, glucose and insulin may also directly exert effects on glucagon secretion from glucagon-secreting α cells (Ronner et al., 1993; Ravier and Rutter, 2005). In contrast, studies by Franklin et al. (2005) demonstrated that in isolated rat α cells, while insulin inhibited glucagon secretion, glucose (16 mM) stimulated glucagon secretion from the rat α cells pretreated with low glucose. The discrepancy is likely due to the use of different assay systems and assay conditions. Furthermore, the modulation of glucagon release may involve

other islet hormones including somatostatin (Cejvan et al., 2003; Hayashi et al., 2003), neurotransmitters such as L-glutamate (Hayashi et al., 2003), or zinc ions (Ishihara et al., 2003). Nevertheless, these studies do not exclude the possibility that the GABA-GABA receptor system might be involved in this modulation (Hayashi et al., 2003; Chessler et al., 2002; Gammelsaeter et al., 2004). These mechanisms appear to constitute precise regulatory networks that operate to regulate glucagon release and hence to maintain blood glucose within a narrow physiological range.

While changing the total number of $GABA_AR$s on the cell surface significantly modulates $GABA_AR$ function (Wang et al., 2003), altering $GABA_AR$ gating or conductance is also important (Mody and Pearce, 2004). It is interesting to note that PKC, a crucial component mediating nutrition-induced insulin resistance in muscle (Kim et al., 2004), fat (Muller et al., 1991), and liver (Lam et al., 2002), is activated and translocated from the cytosol to the plasma membrane following sustained high-fat and/or high-glucose treatment (Lam et al., 2002; Nakajima et al., 2000). Translocation of PKC isoforms to the plasma membrane may allow the direct interaction of PKC and $GABA_AR$ (Brandon et al., 2002). It has been shown previously that direct binding of $GABA_AR$ with activated PKC reduces the amplitude of GABA-activated currents (Connolly et al., 1999; Krishek et al., 1994). It is thus of great interest to investigate the membrane trafficking and the gating of $GABA_AR$ in coupling glucagon secretion under diabetic conditions.

## Experimental procedures

### Cell culture and islet isolation

In-R1-G9 and α-TC6 cells were cultured as described (Drucker et al., 1991; Yamada et al., 2001). Islets were isolated from male Sprague-Dawley rats (200–250 g) and female guinea pigs (200–250 g) by collagenase digestion (Chan et al., 1993). Single islet cells were prepared by dispersion of freshly isolated islets in $Ca^{2+}$-free buffer. Human islets were obtained from the Human Islet Laboratory, University of Pennsylvania. The islets or the dispersed islet cells were maintained in RPMI-1640 tissue culture medium for 2–30 hr prior to the experiments.

### Plasmid construction and transfection

The cDNA of the intracellular loop region between transmembrane domains 3 and 4 of $GABA_AR$ subunits α3, β1, β2, β3, and γ3 were cloned into pGEX-4T-1 for bacterial expression of GST-fusion proteins. Full-length cDNAs for $GABA_AR$ subunits β1, β2, and β3 were cloned into pCMV-Tag 4B for mammalian expression (see Supplemental data). Site-directed mutagenesis was used to introduce S409A in $GABA_AR$-β1, S410A in $GABA_AR$-β2, and S408A/S409A in $GABA_AR$β3 in pGEX-4T and pCMV-Tag 4B, using the Quickchange mutagenesis kit (Stratagene, La Jolla, California). Mammalian cell transfections were carried out using Lipofectamine 2000plus (Invitrogen).

### RT-PCR

One hundred nanograms of RNA template was used to analyze the expression of different $GABA_AR$ subunits using One-step RT-PCR kit (Qiagen, Valencia, CA) in a final volume of 25 μl (see Supplemental data for primer sequences).

### Adenoviral transduction

Adenoviral (Ad) expression vectors tagged with green fluorescent protein (GFP) (Ad-GFP) and carrying wild-type Akt (Ad-wt-Akt), constitutively active (CA) Ad-CA-Akt, or dominant-negative (DN) Akt (Ad-DN-Akt) have been described (Katome et al., 2003). Purified virus was added to ~85% confluent In-R1-G9 or α-TC6 cells for 2 hr. Cells were washed and incubated for 16 hr in fresh medium. Transduction rate was determined by examining the cells for GFP under a fluorescence microscope. With $10^8$ pfu/ml, a transduction rate of >99% was routinely achieved.

### Western blotting and assays of Akt kinase activity

Western blot analysis was performed as described (Wang et al., 2003). The primary antibodies used were anti-IR IgG (1:500), anti-phospho-tyrosine IgG (1:1,000) (Upstate, Lake Placid, New York), anti-Akt IgG (1:1,000), or anti-phospho-Akt (Ser473) (1:500, Cell Signaling Tech, Beverley, Massachusetts). Immunoprecipitation of Akt and the kinase assay were performed using Crosstide (Upstate) as a specific Akt substrate (Wang et al., 1998).

### Phosphorylation assay of GST fusion proteins

Generation of GST-$GABA_AR$ fusion plasmids are described in Supplemental data. In vitro phosphorylation assays were performed as reported (Wang et al., 2003) using recombinant Akt1 kinase (Cell Signaling Technology, Massachusetts).

### Confocal imaging and Cell-ELISA assay

For imaging studies, IN-R1-G9, and α-TC6 cells were grown on poly-D-lysine (Sigma)-coated glass coverslips, while for islets, dispersed rat islet cells were prepared as reported previously (El Kholy et al., 2003). Serum-starved cells were stimulated with insulin (100 nM, 10 min) or vehicle and fixed using 3.7% formaldehyde. After fixation, cells were permeablized with 0.1% Triton X-100, except cells used for translocation studies. Antibodies used were guinea pig anti-insulin, rabbit anti-glucagon, mouse anti-somatostatin (all from Dako, 1:1000), Cy3-, Cy5-, and FITC-conjugated secondary antibodies (Jackson Labs, 1:1000), rabbit anti-$GABA_AR$ $\alpha_1$ subunit (UBI 1:1000), mouse anti-$GABA_AR$ $\beta_{2/3}$ subunit (UBI 1:200), and rabbit anti-phospho-Akt (Ser473, 1:500). Cell-ELISAs were performed as described to quantify the translocation of $GABA_AR$s (Wang et al., 2003). Mouse anti-FLAG (Sigma, 1:800) antibody was used in the cells transfected with various FLAG-tagged $GABA_AR$ vectors as shown in Figure 2C. The dual immunostaining was used to distinguish cell-surface and intracellular $GABA_AR$s. Briefly, for cell-surface labeling, fixed cells were incubated, under nonpermeant conditions, with monoclonal antibody against an extracellular epitope of the $GABA_AR$ $\beta_{2/3}$ subunit (UBI 1:200) and subsequently with Cy3-conjugated anti-mouse IgG (Jackson Labs, 1:1000). Following this, the intracellular receptors were stained, after cell permeabilization, with rabbit anti-$GABA_AR$ $\alpha_1$ subunit (UBI 1:1000) and FITC-conjugated anti-rabbit IgG (Jackson Labs, 1:1000). All multiple stainings were performed sequentially, and individual image channels were scanned separately using a Ziess Laser Scanning Microscope (Model 510). Quantification of surface staining used Metamorph analysis software (Universal Imaging Corp) based on total pixel intensity per unit area. Image thresholds were equal for all experimental conditions, and the background was subtracted from the total number of pixels in the threshold. Total numbers of pixels were then calculated per individual cell. Typically, 100–300 cells were analyzed.

$GABA_AR$ membrane translocation in islet cells was measured using the line profile function of ImagePro Plus 5.0 software (Media Cybernetics Inc., Silver Spring, Maryland) based on fluorescence intensities across single cells along a preset line for each distance increment. Quantification was made from at least 100 cells and expressed as a ratio of membrane/cytosol or membrane/total $GABA_AR$.

### Glucagon and insulin secretion assays

For glucagon secretion assays, the α cells grown in 24-well plates (70%–80% confluency) were washed twice and preincubated with 20 mM glucose in KRB (in mM: 115 NaCl, 5 KCl, 24 $NaHCO_3$, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 0.2% bovine serum albumin) for 30 min at 37°C. Cells were subsequently treated with either low (2 mM) or high (20 mM) glucose in 500 μl KRB containing test substances as indicated. GABA (10 nM) was included in the assay buffer during the incubation period. Islets secretion (10 islets in a 5 ml assay tube) was performed in the KRB buffer except that GABA was omitted and glucose was used in place of insulin. Glucagon and insulin levels were measured by radioimmunoassay (RIA) kits (Linco Research, St. Louis, Missouri). Pilot studies were performed for the time-dependent secretions. Glucagon and insulin were detected within 5 min with a plateau of 15 min with proportional rate of change up to 2–4 hr (data not shown).

### Electrophysiology

Patch-clamp experiments were performed in the perforated whole-cell configuration using an EPC-9 amplifier and Pulse software (HEKA, Lambrecht, Germany). For α-TC6 cells, drugs were applied with computer-controlled

multi-barreled micro-perfusion system (SF-77B, Warner Instruments, Hamden, Connecticut). Intracellular solutions (in mM) contained 130 KCl, 10 NaCl, 0.5 $MgCl_2$, 1 EGTA, 5 HEPES, and 0.15 mg/ml Nystatin (pH 7.3). The standard bath solution (in mM) contained 138 NaCl, 5.6 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 5.6 glucose, 5 HEPES (pH 7.4). For rat $\alpha$ cells, GABA (1 mM) was applied using an oocyte injection system (Nanoliter 2000, World Precision Instruments, Sarasota, Florida). Identification of $\alpha$ cells was based on size and $Na^+$-current inactivation (Göpel et al., 2004). The extracellular solution (in mM) consisted of 118 NaCl, 20 TEACl, 5.6 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, and 5 glucose (pH 7.4). The pipette solution (in mM) contained 50 $Cs_2SO_4$, 60 CsCl, 10 KCl, 1 $MgCl_2$, 10 HEPES and 0.24 mg/ml amphotericin B (pH 7.3). The current responses were recorded at a holding potential of −70 mV. Insulin was added to the bath solution when the responses to two consecutive GABA applications (1 min interval) were stable. For the statistical analysis, the peak current responses were measured.

### Statistical analysis

All data are presented as mean ± SEM. Statistical analysis was done by a Student's t test or ANOVA tests as appropriate. Significance was assumed at a p value of less than 0.05 (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

### Supplemental data

Supplemental data include one figure and supplemental experimental procedures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/3/1/47/DC1/.

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