

# GABA Coordinates with Insulin in Regulating Secretory Function in Pancreatic INS-1 $\beta$ -Cells

Paul Bansal<sup>1,2</sup>, Shuanglian Wang<sup>3,4</sup>, Shenghao Liu<sup>1,2</sup>, Yun-Yan Xiang<sup>3,4</sup>, Wei-Yang Lu<sup>3,4\*</sup>, Qinghua Wang<sup>1,2\*</sup>

**1** Departments of Physiology and Medicine, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada, **2** Division of Endocrinology and Metabolism, The Keenan Research Centre in the Li Ka-Shing Knowledge Institute, St. Michael's Hospital, Toronto, Ontario, Canada, **3** Department of Physiology and Pharmacology, University of Western Ontario, London, Ontario, Canada, **4** Robarts Research Institute, University of Western Ontario, London, Ontario, Canada

## Abstract

Pancreatic islet  $\beta$ -cells produce large amounts of  $\gamma$ -aminobutyric acid (GABA), which is co-released with insulin. GABA inhibits glucagon secretion by hyperpolarizing  $\alpha$ -cells via type-A GABA receptors (GABA<sub>A</sub>Rs). We and others recently reported that islet  $\beta$ -cells also express GABA<sub>A</sub>Rs and that activation of GABA<sub>A</sub>Rs increases insulin release. Here we investigate the effects of insulin on the GABA-GABA<sub>A</sub>R system in the pancreatic INS-1 cells using perforated-patch recording. The results showed that GABA produces a rapid inward current and depolarizes INS-1 cells. However, pre-treatment of the cell with regular insulin (1  $\mu$ M) suppressed the GABA-induced current ( $I_{GABA}$ ) by 43%. Zinc-free insulin also suppressed  $I_{GABA}$  to the same extent of inhibition by regular insulin. The inhibition of  $I_{GABA}$  occurs within 30 seconds after application of insulin. The insulin-induced inhibition of  $I_{GABA}$  persisted in the presence of PI3-kinase inhibitor, but was abolished upon inhibition of ERK, indicating that insulin suppresses GABA<sub>A</sub>Rs through a mechanism that involves ERK activation. Radioimmunoassay revealed that the secretion of C-peptide was enhanced by GABA, which was blocked by pre-incubating the cells with picrotoxin (50  $\mu$ M,  $p < 0.01$ ) and insulin (1  $\mu$ M,  $p < 0.01$ ), respectively. Together, these data suggest that autocrine GABA, via activation of GABA<sub>A</sub>Rs, depolarizes the pancreatic  $\beta$ -cells and enhances insulin secretion. On the other hand, insulin down-regulates GABA-GABA<sub>A</sub>R signaling presenting a feedback mechanism for fine-tuning  $\beta$ -cell secretion.

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\* E-mail: wlu53@uwo.ca (W-YL); qinghua.wang@utoronto.ca (QW)

## Introduction

Gamma-aminobutyric acid (GABA) is a major neurotransmitter in the central nervous system (CNS), where GABA produces fast inhibition in mature neurons primarily by activation of A-type GABA receptor (GABA<sub>A</sub>R), a hetero-pentameric Cl<sup>-</sup> channel [1]. A large amount of GABA is also produced in the pancreatic islet [2], where it exists at the highest concentration outside of the CNS [3]. Pancreatic GABA is primarily produced by the  $\beta$ -cell [4], in which GABA is stored in synaptic-like microvesicles that are distinct from insulin-containing large-dense core vesicles (LDCVs) [5]. However, recent evidence indicates that GABA is co-localized with insulin in LDCVs in human islets and that the release of GABA from the  $\beta$ -cells is glucose-dependent [6]. The release of GABA from  $\beta$ -cells is "tonic" [7,8], yet the amount of released GABA is regulated by the metabolic state of  $\beta$ -cells [9].

In the pancreatic islet, GABA released from  $\beta$ -cells plays a critical role in the regulation of glucagon secretion from  $\alpha$ -cells. Specifically, GABA activates GABA<sub>A</sub>Rs in  $\alpha$ -cells, sequentially leading to an influx of Cl<sup>-</sup> and membrane hyperpolarization, and hence an inhibition of glucagon secretion. The GABA<sub>A</sub>R-mediated hyperpolarization of  $\alpha$ -cells represents a physiological

mechanism for glucose-induced suppression of glucagon release because blockade of GABA<sub>A</sub>R diminishes the inhibitory effect of high glucose on glucagon secretion in isolated rat [10] or mouse [11] islets. In relation to this notion, we have recently demonstrated that insulin suppresses glucagon secretion by enhancing intra-islet GABA-GABA<sub>A</sub>R signaling through translocation of GABA<sub>A</sub>R from an intracellular pool to the cell surface of  $\alpha$ -cells [12].

Studies, including ours, have demonstrated that GABA<sub>A</sub>Rs are also expressed in the primary islet  $\beta$ -cells [12,13] and insulin-secreting clonal  $\beta$ -cell lines [14,15]. Unlike in mature neurons and  $\alpha$ -cells, stimulation of GABA<sub>A</sub>Rs in  $\beta$ -cells induces membrane depolarization, enhancing insulin secretion in the presence of physiological concentrations of glucose [6,15]. Consistent with the notion that the autocrine insulin is essential for  $\beta$ -cell function [16,17], we recently demonstrated that GABA, in cooperation with insulin, enhances the proliferation and survival of the  $\beta$ -cells through activation of the PI3-K/Akt pathway. Remarkably, GABA promotes  $\beta$ -cell regeneration and reverses diabetes in mouse models [18]. In the present study, we found that insulin negatively regulates GABA<sub>A</sub>R function and inhibits GABA-induced  $\beta$ -cell secretion. Our results demonstrated a feedback mechanism that fine-tunes  $\beta$ -cell secretion.

## Materials and Methods

### Cell culture

Rat insulinoma INS-1 cells (passage 50–65) were maintained in RPMI 1640 medium (Invitrogen, Burlington, ON, Canada) containing fetal bovine serum (10% v/v), 100 Units/ml penicillin G sodium, 100  $\mu$ g/ml streptomycin sulphate, 55 mg/500 ml sodium pyruvate, 1.14 g/500 ml HEPES, and 1.7  $\mu$ l/500 ml  $\beta$ -mercaptoethanol at 37°C in an atmosphere of humidified air (95%) and CO<sub>2</sub> (5%). Four hours before being used for patch-clamp recordings, INS-1 cells were glucose-starved in serum-free RPMI 1640 medium that contained 1.4 mM glucose.

### Electrophysiology

For electrophysiological recordings, cells were bathed in the standard extracellular solution (ECS) containing (in mmol/l) 145 NaCl, 1.3 CaCl<sub>2</sub>, 5.4 KCl, 25 HEPES and 1.4 glucose (pH 7.4, 320–340 mOsm), and the ECS was maintained at 30°C. Patch-clamp recordings were performed using an Axopatch-1D amplifier (Axon Instruments, Foster City, CA, USA). Electrodes (1.8–2.3 M $\Omega$ ) were constructed from thin-walled glass (1.5 mm diameter, World Precision Instruments, Sarasota FL, USA) using a two-stage puller (PP-830; Narshige, East Meadow NY, USA). The standard intracellular solution (ICS) consisted of (in mmol/l) 150 KCl, 10 KOH, 10 HEPES, 2 MgCl<sub>2</sub> and 1 CaCl<sub>2</sub> (ATP-free). The pore-forming agent gramicidin (60 mg/ml, Sigma-Aldrich Corp., Buchs, Switzerland) [19] was included in the ICS to perforate the membrane patch of the recorded cell. Under voltage-clamp mode, the membrane perforation was observed as a constant decrease in serial resistance after the electrode seal. In most of the recordings, the resistance declined to a value ranging from 28 to 301 M $\Omega$  within 5–15 min after the seal, and then stabilized for 45–80 min. All perforated patch recordings began when the serial resistance had attained values below 30 M $\Omega$ . To monitor a possible formation of whole-cell configuration, a testing voltage-ramp (a gradual voltage-change from -100 to 100 mV in 1.5 s) was applied to the cell at the start of the recording. With this testing protocol, a sigmoid-shaped current-voltage (I–V) curve was seen under stable perforated patch recordings, whereas a large linear I–V relationship appeared after whole-cell configuration due to the activation of K<sub>ATP</sub> channels by dilution of the cytosolic ATP. The endogenous membrane potential of INS-1 cells was about -60 mV [20]. Thus, INS-1 cells were voltage-clamped at -60 mV while under constant perfusion by fresh ECS. Patch-clamp recording was performed under voltage- or current-clamp mode. Via a computer-controlled multi-barreled perfusion system (SF-77BI Warner Instruments, Hamden, CT, USA), 30  $\mu$ M GABA [the EC<sub>50</sub> of GABA in INS-1 cell is 22.3  $\mu$ M [15]] was briefly (6 s) applied to the patched cells in two-minute intervals. After four stable recordings of GABA-induced current (I<sub>GABA</sub>), insulin was added to the standard ECS. All electrical signals were digitized, filtered (30 kHz), and acquired on-line using the program Clampex and analysed off-line using the program Clampfit 9 (Axon Instruments).

### Measurement of intracellular calcium levels

Cells cultured in 96-well plates were loaded with 5  $\mu$ M Fluo-3 AM (Molecular Probes, Eugene, OR, USA) for 2 hours in Locke's Buffer. Cells were then treated with GABA (30  $\mu$ M), or 5 mM KCl as positive control. Changes in relative fluorescence units (RFU) were monitored with the Fluoroskan Ascent FL fluorescent plate reader equipped with a micro-injection syringe pump (Labsystems, Helsinki, Finland), based on the method provided

by the manufacturer (Molecular Probes and Labsystems Fluo-3 AM Application Note).

### Plasmids transfection

Dominant negative Akt (DN-Akt) vector was constructed as described previously [12]. Green fluorescent protein (GFP)-expressing vector (Invitrogen) was used as an indicator of transfection. INS-1 cells transfected with or without relevant plasmids using Lipofectamine<sup>TM</sup> 2000 (Invitrogen, 24 hrs) according to manufacture's instruction.

### Western blot analysis

Cells were serum-starved (16 hrs) and treated with or without insulin (100 nM) for 10 min, or in the presence of PI3-K inhibitor wortmannin (100 nM) or MEK/ERK inhibitor PD98059 (20  $\mu$ M). Cells were lysed in RIPA lysis buffer containing the protease inhibitors phenylmethylsulphonylfluoride (PMSF) (1 mol/l) and EDTA (1 mol/l), Na<sub>3</sub>VO<sub>4</sub> (1 mol/l), and NaF (1 mol/l). Protein of 25  $\mu$ g was resolved by SDS-PAGE, transferred to nitrocellulose membranes and probed by anti-Akt and anti-phospho-Akt, or anti-ERK1/2 and anti-phospho-ERK1/2 (1:1,000, Cell Signaling) as described previously [12].

### Insulin secretion

INS-1 cells were plated in 24-well plates with a density of  $2.5 \times 10^5$  cells/well in RPMI 1640 medium containing 10% FBS. The following day, the medium was replaced with fresh KRB buffer (containing, in mmol/l, 115 NaCl, 5 KCl, 24 NaHCO<sub>3</sub>, 2.5 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, 1.4 glucose, and 0.1% BSA) for 60 min. The cells were then treated with 1.4 or 11.1 mM glucose in KRB buffer for 2 h, in the presence or absence of GABA (30  $\mu$ M). To determine the effects of insulin and/or GABA on the INS-1 cell secretion, in some parallel assays, cells were pre-treated with insulin (Novolin Toronto, Novo Nordisk, 1  $\mu$ M) or picrotoxin (50  $\mu$ M) for 15 min prior to GABA treatment during the secretion assay. The insulin levels in conditioned KRB buffer were evaluated by measuring C-peptide using a rat C-peptide RIA kit (Linco Research, St. Louis, MO, USA), according to the manufacturer's instructions.

### Confocal Imaging

INS-1 cells were grown on poly-D-lysine (Sigma)-coated 8-well chamber slides (BD Falcon). Serum-starved cells were pre-treated with or without wortmannin (100 nM, 15 min) and incubated with or without insulin (1  $\mu$ M) for 15 min. Cells were fixed with 4% paraformaldehyde and blocked with 2% BSA in PBS containing 0.1% Triton X-100 at room temperature for 1 hour. The cells were then incubated with monoclonal mouse anti-GABA<sub>A</sub>R  $\beta_{2/3}$  subunit (UBI 1:100) and Cy3-conjugated anti-mouse IgG (Jackson Labs, 1:500), consecutively. The images were captured using a Leica TCS 4D laser confocal fluorescence microscope.

### Statistical Analysis

I<sub>GABA</sub> is expressed as mean current normalized to the current amplitude obtained immediately preceding insulin treatment (in all cases the fourth sweep in the series of same-cell current recordings)  $\pm$  SEM. All data were presented as mean  $\pm$  SEM. Statistical analysis was performed using unpaired or paired Student's t-test where appropriate. A *p*-value < 0.05 was considered as significant. Statistical analyses were performed using SigmaPlot 2002 from SPSS Inc. (Chicago, IL, USA) and Microsoft Excel from Microsoft Corp. (Redmond, WA, USA).

## Results

### GABA induces membrane depolarization and increases intracellular $\text{Ca}^{2+}$ levels in INS-1 cells

As previously demonstrated [20], under current-clamp conditions INS-1 cells displayed a quiescent membrane potential around  $-60$  mV when ECS contained  $1.4$  mM glucose (**Figure 1A**). Perfusion of the cell with ECS containing  $28$  mM glucose caused a gradual and sustained depolarization. In some cases, bursts of action potentials were superimposed on the glucose-induced depolarization (**Figure 1A**). Under the same recording conditions, perfusion of GABA induced a fast membrane depolarization in the INS-1 cell (**Figure 1B**). The GABA-induced depolarization was completely blocked by GABA<sub>A</sub>R antagonist picrotoxin (**Figure 1B**) or largely attenuated by bicuculline (not shown), consistent with our previous findings, and those of others in the same cell line or isolated human islet beta  $\beta$ -cells [6,18]. These results suggest that GABA, via activation of GABA<sub>A</sub>R, induces membrane potential depolarization in pancreatic INS-1 cells.

We then performed intracellular  $\text{Ca}^{2+}$  measurements to determine if GABA-induced membrane depolarization increases intracellular  $\text{Ca}^{2+}$  levels in INS-1 cells. As shown in **Figure 1C**, GABA ( $30$   $\mu\text{M}$ ) evoked a steep rise in intracellular  $\text{Ca}^{2+}$  concentrations, which declines and then persists at a stable level during the course of the 20 to 30 min recording period. These results indicate that GABA induces membrane depolarization that is associated with increased intracellular  $\text{Ca}^{2+}$  in a population of pancreatic  $\beta$ -cells.

### Insulin inhibits $I_{\text{GABA}}$ in INS-1 cells

Under voltage-clamp mode, perfusion of GABA evoked typical bicuculline-sensitive GABA current (**Figure 2A**). We next determined the effect of insulin on GABA-induced current ( $I_{\text{GABA}}$ ). Treatment of INS-1 cells with insulin ( $100$  nM) significantly decreased  $I_{\text{GABA}}$  by 22% ( $P < 0.05$ ,  $n = 8$ ). The insulin-induced suppression of  $I_{\text{GABA}}$  was more prominent when the insulin

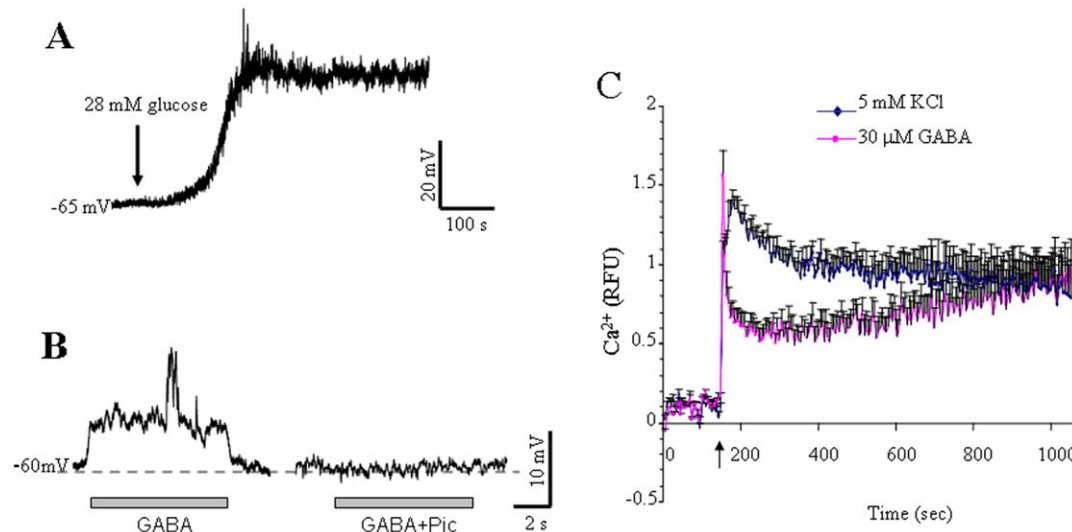
concentration was increased, for instance a reduction of 43% in  $I_{\text{GABA}}$  was achieved with  $1$   $\mu\text{M}$  insulin (**Figure 2A, 2B**,  $p < 0.05$ ,  $n = 5$ ). Thus, insulin-induced suppression of  $I_{\text{GABA}}$  was dose-dependent (**Figure 2C**).

### Zinc-free insulin inhibits $I_{\text{GABA}}$ in INS-1 cells

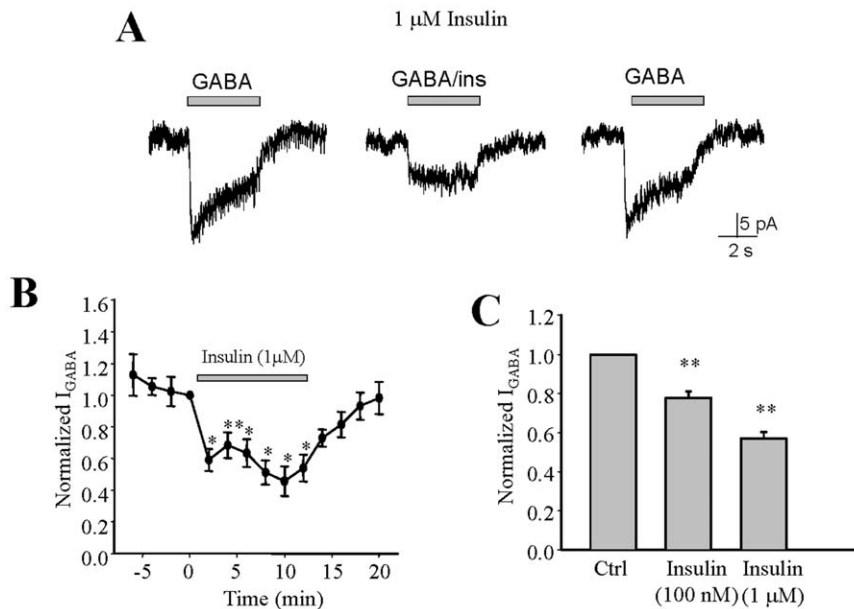
Clinically-used insulin contains zinc [21] and zinc inhibits  $I_{\text{GABA}}$  in neurons [22] by directly binding to GABA<sub>A</sub>R channel and lowering its open probability [23]. Therefore, we used zinc-free insulin to verify the suppressive effects of insulin on  $I_{\text{GABA}}$ . Our result showed that the zinc-free insulin also suppressed  $I_{\text{GABA}}$  in INS-1 cells (**Figure 3A**,  $p < 0.05$ ). The efficacy of zinc-free insulin was similar to that of regular insulin (**Figures 3B and 3C**). However, the suppression of  $I_{\text{GABA}}$  by insulin disappeared when zinc-free insulin was applied simultaneously with GABA (**Figures 3D and 3E**,  $p > 0.05$ ,  $n = 3$ ). We found that the reduction of  $I_{\text{GABA}}$  was seen only when zinc-free insulin was pre-applied to the cell (i.e.,  $>30$  seconds) prior to GABA application (**Figures 3D and 3E**,  $p < 0.05$ ,  $n = 3$ ). These results suggest that zinc-free insulin-induced inhibition of  $I_{\text{GABA}}$  is not a result of direct blockade of GABA<sub>A</sub>R channels, but rather through a signaling process.

### Insulin-induced inhibition on $I_{\text{GABA}}$ is PI3-K/Akt independent

PI3-K is a key signaling molecule that mediates the trophic effects of insulin [24]. We therefore examined whether insulin-induced inhibition of  $I_{\text{GABA}}$  requires involvement of PI3-K. INS-1 cells were pretreated with  $100$  nM of the specific PI3-K inhibitor wortmannin for 10 minutes, and then treated with  $1$   $\mu\text{M}$  zinc-free insulin prior to measurement of  $I_{\text{GABA}}$ . As shown (**Figures 4A and 4B**), the zinc-free insulin-induced inhibition of  $I_{\text{GABA}}$  persisted in the presence of PI3-K inhibitor, which is suggestive of a PI3-K independent process. To confirm this finding, we transfected INS-1 cells with a vector expressing a dominant-negative form of Akt (DN-Akt) and tagged with green fluorescent protein (GFP) [12]. The dominant negative effect of DN-Akt was



**Figure 1. GABA depolarizes membrane potential and increases intracellular  $\text{Ca}^{2+}$  in INS-1 cells.** (A) Perfusion of ECS containing  $28$  mM glucose induces a gradual and sustained depolarization of the membrane potential ( $V_m$ ) ( $n = 5$ ). (B) GABA induces a rapid and GABA<sub>A</sub>R inhibition-sensitive depolarization of  $V_m$  under the current-clamp conditions at  $1.4$  mM glucose ( $n = 5$ ). (C) Cells cultured in 96-well plates pre-loaded with Fluo-3 AM were treated with GABA ( $30$   $\mu\text{M}$ ), or  $5$  mM KCl as positive control. Changes in relative fluorescence units (RFU) were monitored with a fluorescent plate reader. Data are Mean  $\pm$  SE,  $n = 6$ . doi:10.1371/journal.pone.0026225.g001



**Figure 2. GABA-evoked currents ( $I_{GABA}$ ) is inhibited by insulin in INS-1 cells.** GABA-evoked inward current was measured by means of a computer-controlled multi-barrelled perfusion system, in two-minute intervals, under voltage-clamp conditions. Representative traces of GABA-evoked currents in the absence and presence of insulin (100 nM, (A), 1  $\mu$ M, (B)) in the same INS-1 cell. A' and B' represents the average of  $I_{GABA}$  from separated experiments. (C) Normalized average  $I_{GABA}$  during the course of experiment (control = average of first 4  $I_{GABA}$ , insulin = average of  $I_{GABA}$  in the presence insulin at indicated concentrations). Data were mean  $\pm$  SE. \* $p < 0.05$  \*\*  $p < 0.01$ ,  $n = 6$ . doi:10.1371/journal.pone.0026225.g002

determined in parallel experiments by Western Blot using anti-phospho-Akt (Ser473) antibody in either transfected or non-transfected INS-cells treated with or without inhibitors as indicated (**Figure 4C**). Application of zinc-free insulin (1  $\mu$ M) to the transfected INS-1 cells still caused a remarkable reduction of  $I_{GABA}$  (**Figures 4D**). Normalized  $I_{GABA}$  from separated experiments showed zinc-free insulin reduced  $I_{GABA}$  by approximately 30% in INS-1 cells expressing DN-Akt (**Figure 4E**,  $n = 5$ ,  $p < 0.05$ ). These observations suggest that zinc-free insulin-induced inhibition on  $I_{GABA}$  is not sensitive to the PI3-K/Akt inhibition.

#### Insulin does not alter the localization of $GABA_A$ R at the INS-1 plasma membrane

We previously demonstrated that insulin enhances the insertion of  $GABA_A$ R into the plasma membrane in neuron [25] and  $\alpha$ -cell [12]. We thus investigated whether insulin could alter  $GABA_A$ R membrane expression in INS-1 cells by immunostaining using antibody against the  $GABA_A$ R $\beta_{2/3}$  subunits. As shown, insulin (1  $\mu$ M, 15 min) did not alter the staining profile of  $GABA_A$ R $\beta_{2/3}$  subunits (**Figure 5A**) in INS-1 cells treated with or without wortmannin, suggesting that insulin-induced suppression of  $I_{GABA}$  is not related to  $GABA_A$ R redistribution in INS-1 cells.

#### Insulin-induced inhibition of $I_{GABA}$ is ERK dependent

Activation of the MRK/ERK pathway represents another important branch of the insulin receptor signal transduction pathway in pancreatic  $\beta$ -cells [26]. We performed experiments to examine whether or not insulin-induced inhibition of  $I_{GABA}$  requires activation of MEK/ERK. We found that incubation of INS-1 cells with insulin (100 nM, 5 min) resulted in rapid ERK1/2 phosphorylation, which was blocked by the MEK/ERK inhibitor PD98059 (20  $\mu$ M) (**Figure 5B**). Perforated patch clamp recordings were then performed in the control INS-1 cells and INS-1 cells that were pretreated with PD98059

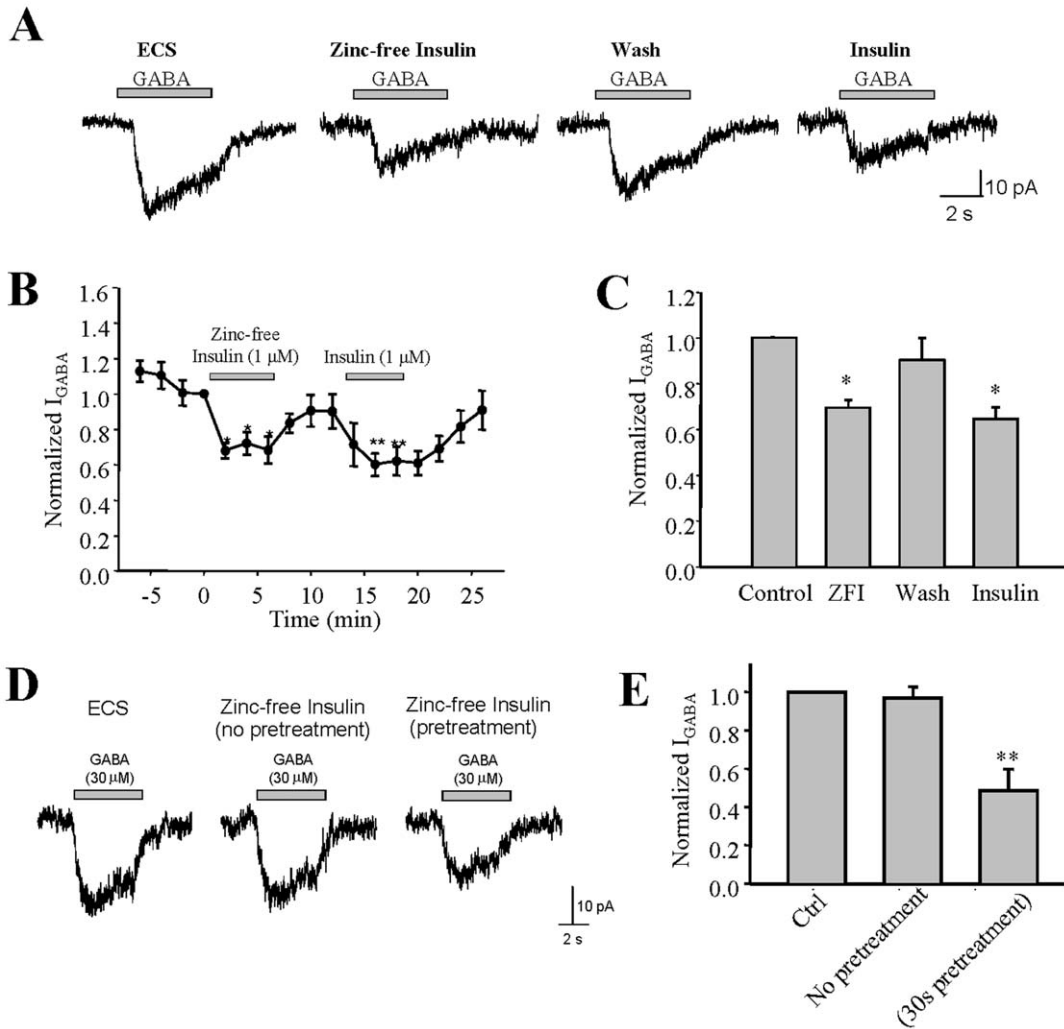
(20  $\mu$ M, 10 min). We found that application of zinc-free insulin to the cells failed to inhibit  $I_{GABA}$  in the presence of PD98059 (**Figures 5C, 5D**;  $p < 0.05$ ,  $n = 6$ ). This result suggests that insulin suppresses  $GABA_A$ R function via activation of the MEK/ERK pathway.

#### Insulin inhibits GABA-induced INS-1 cell secretion

We next conducted C-peptide radioimmunoassays (RIA) to determine whether or not the effect of insulin on the modulation of GABA- $GABA_A$ R system has an impact on  $GABA_A$ R-mediated secretory function in INS-1 cells. As shown, consistent with our previous findings [15], GABA (30  $\mu$ M) significantly increased C-peptide secretion in INS-1 cells, which was diminished by the  $GABA_A$ R antagonist picrotoxin (50  $\mu$ M) (**Figure 6**,  $p < 0.01$ ,  $n = 3$ ), suggesting that GABA-induced insulin secretion in the  $\beta$ -cells is mediated by  $GABA_A$ R. Furthermore, pre-treatment of the INS-1 cells with insulin (1  $\mu$ M) resulted in a statistically-significant decrease in GABA-induced C-peptide secretion (**Figure 6**,  $p < 0.01$ ,  $n = 3$ ). These results suggest that insulin-mediated inhibition of  $I_{GABA}$  is related to down-regulation in GABA-induced insulin secretion in INS-1 cells.

#### Discussion

Pancreatic  $\beta$ -cells produce a large amount of GABA [27], whereas  $GABA_A$ Rs are expressed in both  $\beta$ -cells [6,15] and  $\alpha$ -cells [7,12]. In  $\alpha$ -cells, GABA hyperpolarizes the membrane potential and suppresses glucagon secretion [7,12], via a mechanism involving PI3-K/Akt signaling dependent  $GABA_A$ R plasma membrane translocation [12]. In contrast, we and others demonstrated that GABA depolarizes  $\beta$ -cells and stimulates insulin secretion from these cells [6,15]. These observations suggest that GABA, as a paracrine or autocrine factor plays an important role within pancreatic islets in the regulation of islet cell secretion and function. In the present study, we sought to



**Figure 3. Zinc-free insulin inhibits GABA-evoked currents in INS-1 cells.** (A) Representative traces of GABA-evoked currents in the absence and presence of zinc-free insulin and regular insulin in the same INS-1 cell. (B) The average of  $I_{GABA}$  from separated experiments. (C) Normalized average  $I_{GABA}$  was separately recorded during the course of experiment (control = average of first 4  $I_{GABA}$ , ZFI = average of  $I_{GABA}$  in the presence zinc-free insulin, insulin = average of  $I_{GABA}$  in the presence of insulin after washing out). (D) Representative traces of  $I_{GABA}$  obtained when GABA was applied simultaneously with insulin or 30 seconds after insulin pre-treatment. (E) Normalized average  $I_{GABA}$  of separated experiments as described in (C). Data were mean  $\pm$  SE. \* $p < 0.05$ ,  $n = 5$ . doi:10.1371/journal.pone.0026225.g003

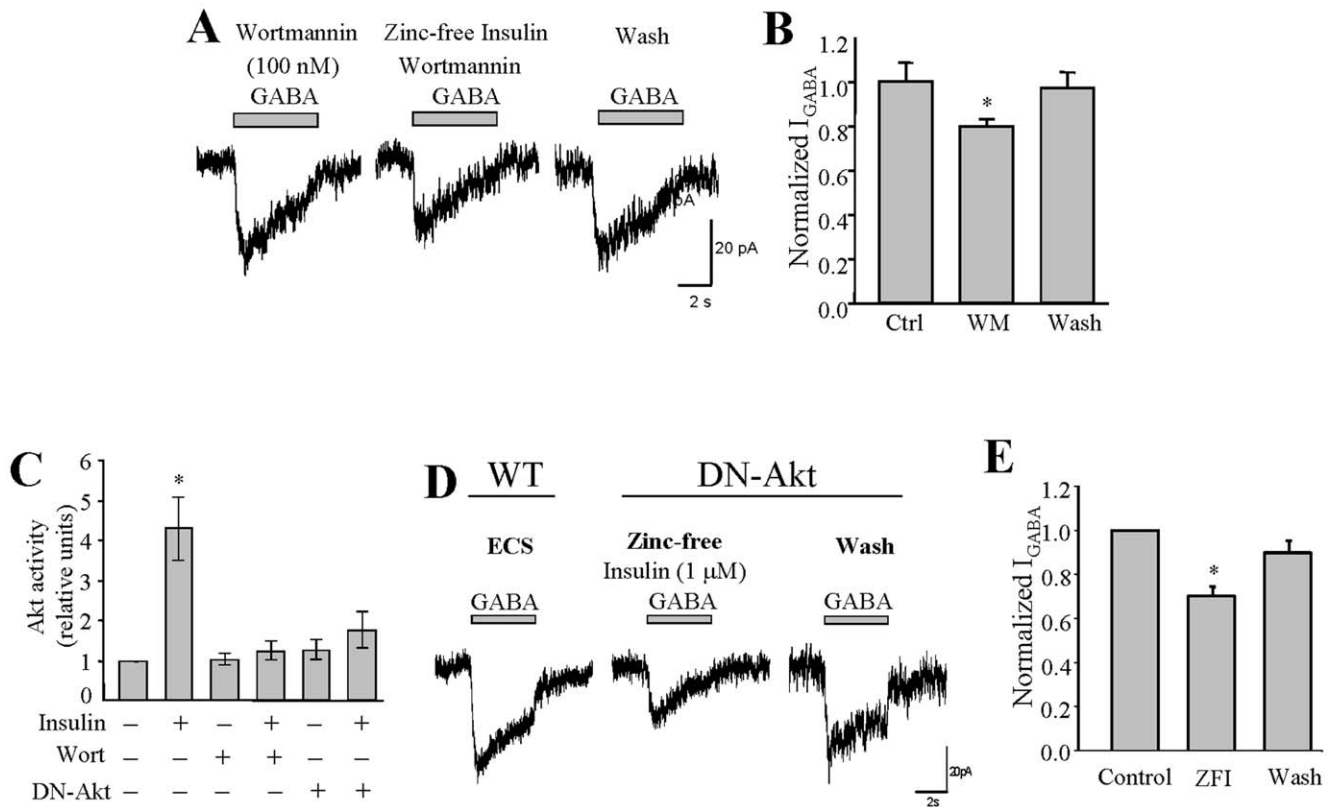
investigate how insulin affects GABA-GABA<sub>A</sub>R system in the  $\beta$ -cells and thereby modulates its secretory pathways.

In INS-1 cells, glucose induces a gradual and sustained depolarization, whereas GABA produces rapid and bicuculline- or picrotoxin-sensitive membrane depolarization, associated with remarkable increases in intracellular  $Ca^{2+}$  concentration and insulin secretion. A recent study by Braun *et al.* suggested that glucose stimulates feed-forward release of GABA from the  $\beta$ -cells [6]. Furthermore, the GABA-stimulated insulin release appears to be glucose concentration-dependent [6,15]. Of note, our results showed that GABA<sub>A</sub>R antagonist picrotoxin attenuated about 50% of the GABA-induced C-peptide release. This is likely due to the fact that GABA-stimulated insulin secretion in the  $\beta$ -cells is partially contributed by activation of B-type GABA receptor (GABA<sub>B</sub>R) [28]. These observations suggest that the autocrine GABA-GABA<sub>A</sub>R system in  $\beta$ -cells constitutes an effective signaling component of the glucose-sensing machinery.

The opposite effects of GABA in the two types of islet endocrine cells are likely because  $\beta$ -cells and  $\alpha$ -cells have different  $Cl^-$

reversal potential ( $E_{Cl}$ ). The direction of  $Cl^-$  flow upon opening of the GABA<sub>A</sub>R channel is dependent on the electrochemical driving force which is determined by the resting membrane potential and the  $E_{Cl}$  [29]. For example, in the early developing brain, GABA induces depolarizing effects in immature neurons [30], while it exerts inhibitory effects by hyperpolarizing the membrane potential in mature neurons of the adult brain [31]. The switch from excitation to inhibition of GABA<sub>A</sub>R activation is due to a shift of  $E_{Cl}$  which is controlled by increased activity of  $K^+$ - $Cl^-$  co-transporter-2 (KCC2) in the brain during development [32]. In this regard, functional KCC has been identified in pancreatic  $\alpha$ -cells, but not in the  $\beta$ -cells [33,34].

Regular human insulin is a complex of insulin and zinc [35]. The finding that zinc-free insulin suppressed  $I_{GABA}$  to a degree similar to that of regular insulin suggests that the inhibitory effects of insulin on  $I_{GABA}$  is dependent on the insulin peptide. It is interesting to note that application of zinc-free insulin together with GABA did not inhibit  $I_{GABA}$ , whereas pre-treating the cell for at least 30 seconds with zinc-free insulin inhibited  $I_{GABA}$ . These



**Figure 4. Insulin-induced inhibition of  $I_{GABA}$  in INS-1 cells is PI3-K/Akt independent.** (A) Representative traces of GABA-evoked currents in the absence and presence of zinc-free insulin (1  $\mu$ M) along with PI3-K inhibitor wortmannin (100 nM). (B) Normalized average of  $I_{GABA}$  from separated experiments. (C) Akt activity determined by Western Blotting using anti-phospho Akt (S473) in cells treated without or with wortmannin (Wort), or in the cells transfected with dominant-negative Akt (DN-Akt). (D) Representative traces of GABA-evoked currents in cells expressing DN-Akt in the absence and presence of zinc-free insulin (ZFI, 1  $\mu$ M). (E) Average  $I_{GABA}$  from separated time-course experiments. Data were mean  $\pm$  SE. \* $p < 0.05$ , \*\*  $p < 0.01$ ,  $n = 5$ .

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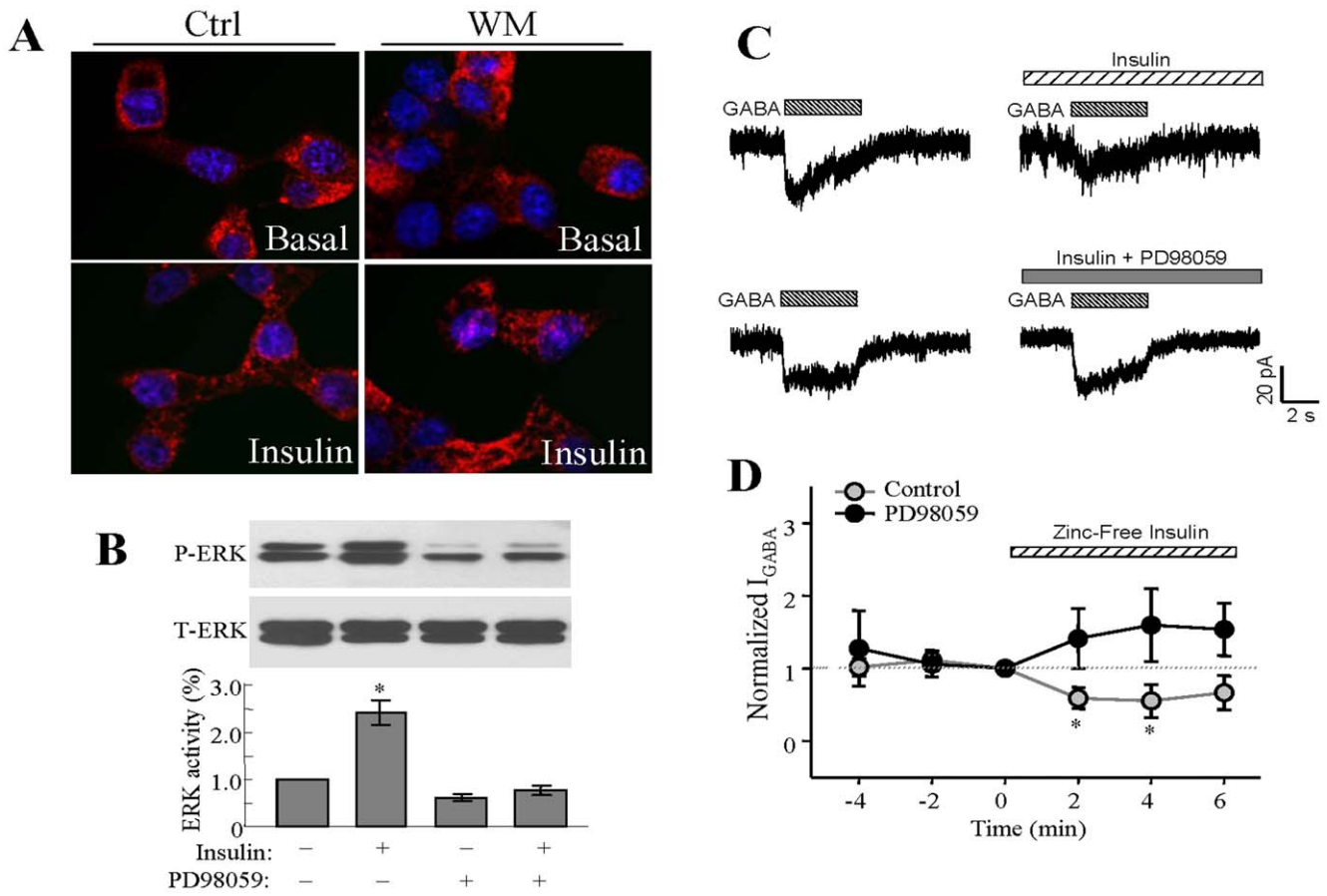
results suggest that insulin-induced inhibition of  $I_{GABA}$  in INS-1 cells requires insulin signaling processes. Such inhibitory effects of insulin on GABA-induced current was also observed in the non-islet  $\beta$ -cells [36]. The potentiating effect of insulin on  $I_{GABA}$  in neurons and  $\alpha$ -cells is attributed to  $GABA_{A,R}$  insertion into the plasma membrane, which occurred about 10-15 min after insulin treatment [12,25]. Under similar experimental conditions, however, we did not observe increased  $GABA_{A,R}$  localization at the plasma membrane upon insulin treatment in INS-1 cells. Furthermore, unlike in the  $\alpha$ -cells, where the insulin-enhanced  $I_{GABA}$  is PI3-K/Akt dependent, our data does not suggest the involvement of PI3-K/Akt signaling in the inhibition of  $I_{GABA}$  by insulin in the  $\beta$ -cells. In contrast, MEK/ERK inhibitor PD98059 blocked the inhibitory effect of insulin on GABA-induced current, suggesting that insulin regulates  $GABA_{A,R}$  function in INS-1 cells via activation of the MEK/ERK signaling pathway.

The explanation for the opposite effects of insulin on  $GABA_{A,R}$  in  $\alpha$ - and  $\beta$ -cells is largely unknown, although it may be due to the different subunit composition of  $GABA_{A,R}$  in the two types of islet cells [12,15]. It is interesting to note that in neurons, activation of the insulin-PI3-K signaling pathway enhances  $I_{GABA}$  due to the increase in cell surface-localized  $GABA_{A,R}$ , whereas activation of insulin receptor with ERK kinase causes inhibition of  $I_{GABA}$  through phosphorylation of a specific subunit of  $GABA_{A,R}$  [36,37]. Particularly,  $\alpha$ -subunits of the  $GABA_{A,R}$  have a putative phosphorylation site for ERK [37]. Presumably, such phosphor-

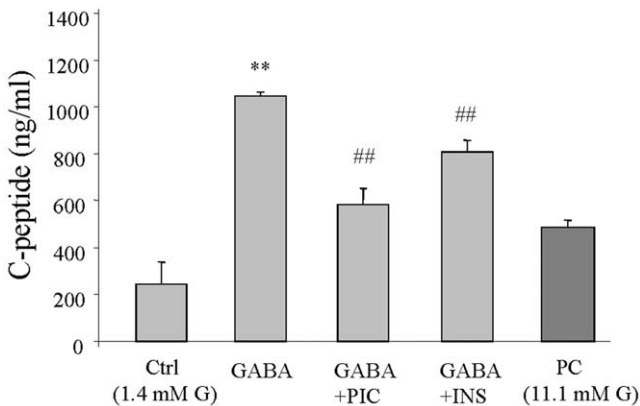
ylation occurs on an intracellular site allowing immediate allosteric modifications of  $GABA_{A,R}$ .

Given the relatively rapid inhibitory effect of insulin on  $I_{GABA}$ , it is possible that insulin may also act as a non-competitive inhibitor of the  $GABA_{A,R}$  in the  $\beta$ -cells, as has been observed in non- $\beta$ -cells [36]. In relation to this notion, it has been reported that a direct receptor-receptor interaction occurs between  $GABA_{A,R}$  and dopamine D5 receptor, which affects the  $GABA_{A,R}$  activation [38]. Further study is warranted to test if there is an interaction between insulin receptor and  $GABA_{A,R}$ , and to determine the molecular mechanism by which insulin modulates  $GABA_{A,R}$  signaling in the  $\beta$ -cells.

In INS-1 cells, insulin suppresses  $I_{GABA}$  and decreases GABA-mediated insulin secretion in the  $\beta$ -cells which suggests that insulin may utilize the GABA- $GABA_{A,R}$  system to constitute a feedback mechanism for the  $\beta$ -cell secretion. Our findings are in a good agreement with previous observations suggesting that activation of insulin receptor inhibits insulin secretion in the  $\beta$ -cells [39]. Conversely, inhibition of PI3-K signaling pathways enhances insulin secretion in the  $\beta$ -cells [40,41]. A study by Khan et al suggested that insulin inhibits insulin secretion through activation of  $K_{ATP}$  channels in the  $\beta$ -cells [42]. A study by Jimenez-Felstrom and colleagues [43] suggested that the effect of insulin on  $I_{GABA}$  is insulin-dose dependent, exemplified by the observation that, insulin, at low concentrations (i.e., from 0.05 to 0.1 nM) stimulated insulin release, while at concentra-



**Figure 5. Insulin suppresses  $I_{GABA}$  which is not associated with GABA<sub>A</sub>R membrane relocation and is ERK-dependent.** (A) Confocal microscopic image of INS-1 cells immunostained for GABA<sub>A</sub>R using anti-GABA<sub>A</sub>R  $\beta_{2/3}$  mouse IgG and Cy3-conjugated secondary antibody (red) with DAPI-nuclear staining (blue). Cells were treated with or without insulin, in the presence or absence of PI3-K inhibitor wortmannin. (B) Insulin (100 nM, 5 min) stimulated ERK phosphorylation in INS-1 cells, which was blocked by pre-treatment of the cells with PD98059 (20  $\mu$ M, 10 min). (C) Representative traces of GABA-evoked currents in the absence and presence of zinc-free insulin (0.6  $\mu$ M) with or without PD98059 (20  $\mu$ M). (D) Normalized average of  $I_{GABA}$  from separated experiments. Data were mean  $\pm$  SE. \* $p < 0.05$ , \*\*  $p < 0.01$ ,  $n = 5-6$ . doi:10.1371/journal.pone.0026225.g005



**Figure 6. GABA enhances insulin secretion which is attenuated by insulin in INS-1 cells.** Insulin secretion was evaluated by C-peptide RIA. Cells were serum-starved in KRB buffer containing 1.4 mM glucose for 60 min prior to the RIA. The RIA was conducted using cells which had their culture medium replaced with fresh KRB buffer containing 1.4 mM glucose (or 11.1 mM glucose as positive control, PC), in the presence of GABA (30  $\mu$ M), with or without either picrotoxin (Pic, 50  $\mu$ M) or insulin (Ins, 1  $\mu$ M) for 120 min. Data were mean  $\pm$  SE, from three independent experiments with each sample counted in triplication. \*\*  $p < 0.01$  (Ctrl vs GABA), ##  $p < 0.01$  (GABA vs GABA+Pic or GABA+Ins). doi:10.1371/journal.pone.0026225.g006

tions higher than 250 nM, insulin inhibited insulin secretion from the  $\beta$ -cells.

It should be noted that under certain circumstance, effects of insulin on  $I_{GABA}$  are excitatory [16]. These previous reports that describe the stimulatory effects of insulin on  $\beta$ -cell secretory process were mostly supported by experiments involving  $\beta$ -cells from organisms with genetic knockout or overexpression of the insulin receptor [44–48]. The different outcomes imply that the modulation of insulin on the GABA-GABA<sub>A</sub>R system in the  $\beta$ -cells may be dependent on their metabolic status.

The physiological relevance of GABA signaling in the regulation of islet  $\beta$ -cell function has yet to be fully identified. We demonstrated recently that the depolarizing effects of GABA may lead to activation of PI3-K/Akt dependent cell growth and survival pathways in the  $\beta$ -cells [18]. Insulin is an important positive autocrine regulator of  $\beta$ -cell growth and survival [17,49]. GABA, when co-released with insulin [6], synergistically enhances insulin-stimulated cell growth and survival pathways in the  $\beta$ -cells [18]. In support of previous findings that insulin is a negative regulator of insulin secretion [39–42,50], our data suggest that insulin utilizes the autocrine GABA-GABA<sub>A</sub>R pathway to operate its negative feedback suppression in the  $\beta$ -cells.

Such a negative feedback modulator appears to be important for maintaining islet hormones at appropriate levels [51]. It is

conceivable that basal insulin may serve as a maintenance signal that primes the  $\beta$ -cell to respond to subsequent glucose stimulus, insulin may utilize GABA-GABA<sub>A</sub>R system to inhibit further release at the peak of the exocytotic event, particularly, at very high local insulin concentration. Previous euglycemic hyperinsulinemic clamp studies in humans suggest that this negative short-loop insulin- $\beta$ -cell feedback is an important mechanism in maintaining appropriate  $\beta$ -cell secretion, since inadequate feedback suppression is found in obese patients, and may partly account for their prevailing hyperinsulinemia [52]. Given that autocrine insulin action is critical in maintaining normal  $\beta$ -cell function [16,17], and that  $\beta$ -cell insulin resistance can deteriorate  $\beta$ -cell function that accelerates the progression of diabetes [53,54], future studies are required to determine whether the impairment

of the autocrine insulin-GABA-GABA<sub>A</sub>R signaling contributes to  $\beta$ -cell insulin resistance in type 2 diabetes.

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## Author Contributions

Conceived and designed the experiments: PB WL QW. Performed the experiments: PB YX SW SL. Analyzed the data: PB WL QW SW SL. Wrote the paper: PB WL QW.

## References

- Luscher B, Keller CA (2004) Regulation of GABAA receptor trafficking, channel activity, and functional plasticity of inhibitory synapses. *Pharmacol Ther* 102: 195–221.
- Okada Y, Taniguchi H, Shimada C (1976) High Concentration of GABA and High Glutamate Decarboxylase Activity in Rat Pancreatic Islets and Human Insulinoma. *Science* 194: 620–622.
- Gerber JC, III, Hare TA (1979) Gamma-aminobutyric acid in peripheral tissue, with emphasis on the endocrine pancreas: presence in two species and reduction by streptozotocin. *Diabetes* 28: 1073–1076.
- Vincent SR, Hokfelt T, Wu JY, Elde RP, Morgan LM, et al. (1983) Immunohistochemical studies of the GABA system in the pancreas. *Neuroendocrinology* 36: 197–204.
- Reetz A, Solimena M, Matteoli M, Folli F, Takei K, et al. (1991) GABA and pancreatic beta-cells: colocalization of glutamic acid decarboxylase (GAD) and GABA with synaptic-like microvesicles suggests their role in GABA storage and secretion. *EMBO J* 10: 1275–1284.
- Braun M, Ramracheya R, Bengtsson M, Clark A, Walker JN, et al. (2010) Gamma-Aminobutyric Acid (GABA) Is an Autocrine Excitatory Transmitter in Human Pancreatic Beta-Cells. *Diabetes* 59: 1694–1701.
- Rorsman P, Berggren PO, Bokvist K, Ericson H, Mohler H, et al. (1989) Glucose-inhibition of glucagon secretion involves activation of GABAA-receptor chloride channels. *Nature* 341: 233–236.
- Smismans A, Schuit F, Pipeleers D (1997) Nutrient regulation of gamma-aminobutyric acid release from islet beta cells. *Diabetologia* 40: 1411–1415. doi: 10.1007/s001250050843.
- Winnock F, Ling Z, De Proft R, Dejonghe S, Schuit F, et al. (2002) Correlation between GABA release from rat islet beta -cells and their metabolic state. *Am J Physiol Endocrinol Metab* 282: E937–E942.
- Wendt A, Birnir B, Buschard K, Gromada J, Salehi A, et al. (2004) Glucose Inhibition of Glucagon Secretion From Rat [alpha]-Cells Is Mediated by GABA Released From Neighboring [beta]-Cells. *Diabetes* 53: 1038–1045.
- Bailey SJ, Ravier MA, Rutter GA (2007) Glucose-dependent regulation of gamma-aminobutyric acid (GABA A) receptor expression in mouse pancreatic islet alpha-cells. *Diabetes* 56: 320–327.
- Xu E, Kumar M, Zhang Y, Ju W, Obata T, et al. (2006) Intra-islet insulin suppresses glucagon release via GABA-GABAA receptor system. *Cell Metab* 3: 47–58.
- Glassmeier G, Hopfner M, Buhr H, Lemmer K, Riecken EO, et al. (1998) Expression of functional GABAA receptors in isolated human insulinoma cells. *Ann N Y Acad Sci* 859: 241–248.
- Blankenfeld G, Enkvist MOK, Kettenmann H, Turner J, Ahnert-Hilger G, et al. (1995) Expression of functional GABAA receptors in neuroendocrine gastropancreatic cells. *Pflügers Archiv European Journal of Physiology* 430: 381–388. doi: 10.1007/BF00373913.
- Dong H, Kumar M, Zhang Y, Gyulkhandanyan A, Xiang YY, et al. (2006) Gamma-aminobutyric acid up- and downregulates insulin secretion from beta cells in concert with changes in glucose concentration. *Diabetologia* 49: 697–705.
- Leibiger IB, Leibiger B, Berggren PO (2008) Insulin Signaling in the Pancreatic [beta]-Cell. *Annual Review of Nutrition* 28: 233–251. doi: 10.1146/annurev-nutr.28.061807.155530.
- Wang Q, Jin T (2009) The role of insulin signaling in the development of beta-cell dysfunction and diabetes. *Islets* 1: 95–101.
- Soltani N, Qiu H, Aleksic M, Glinka Y, Zhao F, et al. (2011) GABA exerts protective and regenerative effects on islet beta cells and reverses diabetes. *Proc Natl Acad Sci U S A* 108: 11692–11697.
- Sugita M, Hirono C, Shiba Y (2004) Gramicidin-perforated Patch Recording Revealed the Oscillatory Nature of Secretory Cl- Movements in Salivary Acinar Cells. *The Journal of General Physiology* 124: 59–69.
- Li J, Zhang N, Ye B, Ju W, Orser B, et al. (2007) Non-steroidal anti-inflammatory drugs increase insulin release from beta cells by inhibiting ATP-sensitive potassium channels. *Br J Pharmacol* 151: 483–493. doi: 10.1038/sj.bjp.0707259 [pii];10.1038/sj.bjp.0707259 [doi].
- Smith GD, Swenson DC, Dodson EJ, Dodson GG, Reynolds CD (1984) Structural stability in the 4-zinc human insulin hexamer. *Proc Natl Acad Sci U S A* 81: 7093–7097.
- Aguayo LG, Alarcon JM (1993) Modulation of the developing rat sympathetic GABAA receptor by Zn<sup>++</sup>, benzodiazepines, barbiturates and ethanol. *Journal of Pharmacology and Experimental Therapeutics* 267: 1414–1422.
- Hosie AM, Dunne EL, Harvey RJ, Smart TG (2003) Zinc-mediated inhibition of GABAA receptors: discrete binding sites underlie subtype specificity. *Nat Neurosci* 6: 362–369. doi: 10.1038/nn1030.
- Taniguchi CM, Emanuelli B, Kahn CR (2006) Critical nodes in signalling pathways: insights into insulin action. *Nat Rev Mol Cell Biol* 7: 85–96. doi: 10.1038/nrm1837.
- Wang Q, Liu L, Pei L, Ju W, Ahmadian G, et al. (2003) Control of Synaptic Strength, a Novel Function of Akt. *Neuron* 38: 915–928. doi: DOI: 10.1016/S0896-6273(03)00356-8.
- Assmann A, Ueki K, Winnay JN, Kadowaki T, Kulkarni RN (2009) Glucose effects on beta-cell growth and survival require activation of insulin receptors and insulin receptor substrate 2. *Mol Cell Biol* 29: 3219–3228.
- Adegate E, Ponery AS (2002) GABA in the endocrine pancreas: cellular localization and function in normal and diabetic rats. *Tissue and Cell* 34: 1–6. doi: DOI: 10.1054/tice.2002.0217.
- Ligon B, Yang J, Morin SB, Ruberti MF, Steer ML (2007) Regulation of pancreatic islet cell survival and replication by gamma-aminobutyric acid. *Diabetologia* 50: 764–773.
- Bormann J (1988) Electrophysiology of GABAA and GABAB receptor subtypes. *Trends in Neurosciences* 11: 112–116. doi: DOI: 10.1016/0166-2236(88)90156-7.
- Chen G, Trombley PQ, van den Pol AN (1996) Excitatory actions of GABA in developing rat hypothalamic neurons. *The Journal of Physiology* 494: 451–464.
- Owens DF, Kriegstein AR (2002) Is there more to gaba than synaptic inhibition? *Nat Rev Neurosci* 3: 715–727. doi: 10.1038/nrn919.
- Ludwig A, Li H, Saarma M, Kaila K, Rivera C (2003) Developmental up-regulation of KCC2 in the absence of GABAergic and glutamatergic transmission. *Eur J Neurosci* 18: 3199–3206. doi: 10.1038/15113.
- Davies SL, Roussa E, Le Rouzic P, Thevenod F, Alper SL, et al. (2004) Expression of K<sup>+</sup>-Cl<sup>-</sup> cotransporters in the [alpha]-cells of rat endocrine pancreas. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1667: 7–14. doi: DOI: 10.1016/j.bbame.2004.08.005.
- Takayama C, Inoue Y (2010) Developmental localization of potassium chloride co-transporter 2 (KCC2), GABA and vesicular GABA transporter (VGAT) in the postnatal mouse somatosensory cortex. *Neurosci Res* 67: 137–148.
- Chausmer AB (1998) Zinc, Insulin and Diabetes. *J Am Coll Nutr* 17: 109–115.
- Williams DB (2008) A novel, rapid, inhibitory effect of insulin on [alpha]1[beta]2[gamma]2s [gamma]-aminobutyric acid type A receptors. *Neuroscience Letters* 443: 27–31. doi: DOI: 10.1016/j.neulet.2008.07.038.
- Bell-Horner CL, Dohi A, Nguyen Q, Dillon GH, Singh M (2006) ERK/MAPK pathway regulates GABAA receptors. *J Neurobiol* 66: 1467–1474.
- Liu F, Wan Q, Pristupa ZB, Yu XM, Wang YT, et al. (2000) Direct protein-protein coupling enables cross-talk between dopamine D5 and [gamma]-aminobutyric acid A receptors. *Nature* 403: 274–280. doi: 10.1038/35002014.
- Persaud SJ, Asare-Anane H, Jones PM (2002) Insulin receptor activation inhibits insulin secretion from human islets of Langerhans. *FEBS Letters* 510: 225–228. doi: DOI: 10.1016/S0014-5793(01)03268-9.
- Collier JJ, White SM, Dick GM, Scott DK (2004) Phosphatidylinositol 3-kinase inhibitors reveal a unique mechanism of enhancing insulin secretion in 832/13 rat insulinoma cells. *Biochem Biophys Res Commun* 324: 1018–1023.
- Eto K, Yamashita T, Tsubamoto Y, Terauchi Y, Hirose K, et al. (2002) Phosphatidylinositol 3-kinase suppresses glucose-stimulated insulin secretion by affecting post-cytosolic [Ca<sup>2+</sup>] elevation signals. *Diabetes* 51: 87–97.



42. Khan FA, Goforth PB, Zhang M, Satin LS (2001) Insulin Activates ATP-Sensitive K<sup>+</sup> Channels in Pancreatic  $\beta$ -Cells Through a Phosphatidylinositol 3-Kinase-Dependent Pathway. *Diabetes* 50: 2192–2198.
43. Jimenez-Felstrom J, Lundquist I, Obermuller S, Salehi A (2004) Insulin feedback actions: complex effects involving isoforms of islet nitric oxide synthase. *Regul Pept* 122: 109–118.
44. Aspinwall CA, Lakey JRT, Kennedy RT (1999) Insulin-stimulated Insulin Secretion in Single Pancreatic Beta Cells. *J Biol Chem* 274: 6360–6365.
45. Aspinwall CA, Qian WJ, Roper MG, Kulkarni RN, Kahn CR, et al. (2000) Roles of Insulin Receptor Substrate-1, Phosphatidylinositol 3-Kinase, and Release of Intracellular Ca<sup>2+</sup> Stores in Insulin-stimulated Insulin Secretion in  $\beta$ -Cells. *J Biol Chem* 275: 22331–22338.
46. Kulkarni RN, Bruning JC, Winnay JN, Postic C, Magnuson MA, et al. (1999) Tissue-Specific Knockout of the Insulin Receptor in Pancreatic  $\beta$  Cells Creates an Insulin Secretory Defect Similar to that in Type 2 Diabetes. *Cell* 96: 329–339. doi: DOI: 10.1016/S0092-8674(00)80546-2.
47. Otani K, Kulkarni RN, Baldwin AC, Krutzfeldt J, Ueki K, et al. (2004) Reduced  $\beta$ -cell mass and altered glucose sensing impair insulin-secretory function in  $\beta$ -IRKO mice. *Am J Physiol Endocrinol Metab* 286: E41–E49.
48. Xu GG, Rothenberg PL (1998) Insulin receptor signaling in the beta-cell influences insulin gene expression and insulin content: evidence for autocrine beta-cell regulation. *Diabetes* 47: 1243–1252.
49. Bansal P, Wang Q (2008) Insulin as a physiological modulator of glucagon secretion. *Am J Physiol Endocrinol Metab* 295: E751–E761.
50. Elahi D, Nagulesparan M, Hershcopf RJ, Muller DC, Tobin JD, et al. (1982) Feedback inhibition of insulin secretion by insulin: relation to the hyperinsulinemia of obesity. *N Engl J Med* 306: 1196–1202.
51. Zhang Y, Zhang N, Gyulkhandanyan A, Xu E, Gaisano H, et al. (2008) Presence of functional hyperpolarisation-activated cyclic nucleotide-gated channels in clonal alpha cell lines and rat islet alpha cells. *Diabetologia* 51: 2290–2298.
52. Elahi D, Nagulesparan M, Hershcopf RJ, Muller DC, Tobin JD, et al. (1982) Feedback inhibition of insulin secretion by insulin: relation to the hyperinsulinemia of obesity. *N Engl J Med* 306: 1196–1202.
53. Ueki K, Okada T, Hu J, Liew CW, Assmann A, et al. (2006) Total insulin and IGF-I resistance in pancreatic  $\beta$  cells causes overt diabetes. *Nat Genet* 38: 583–588. doi: 10.1038/ng1787.
54. Zhao J, Zhang N, He M, Yang Z, Tong W, et al. (2008) Increased Beta-Cell Apoptosis and Impaired Insulin Signaling Pathway Contributes to the Onset of Diabetes in OLETF Rats. *Cellular Physiology and Biochemistry* 21: 445–454.