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Gamma-aminobutyric acid up- and downregulates insulin secretion from beta cells in concert with changes in glucose concentration

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Abstract *Aims/hypothesis:* The role of gamma-aminobutyric acid (GABA) and A-type GABA receptors (GABA_ARs) in modulating islet endocrine function has been actively investigated since the identification of GABA and GABA_ARs in the pancreatic islets. However, the reported effects of GABA_AR activation on insulin secretion from islet beta cells have been controversial. *Methods:* This study examined the hypothesis that the effect of GABA on beta cell insulin secretion is dependent on glucose concentration. *Results:* Perforated patch-clamp recordings in INS-1 cells demonstrated that GABA, at concentrations ranging from 1 to 1,000 μmol/l, induced a transmembrane current (I_{GABA}) which was sensitive to the GABA_AR antagonist bicuculline. The current–voltage relationship revealed that I_{GABA} reversed at -42 ± 2.2 mV, independently of glucose concentration. Nevertheless, the glucose concentration critically controlled the membrane potential (V_M), i.e., at low glucose (0 or 2.8 mmol/l) the

endogenous V_M of INS-1 cells was below the I_{GABA} reversal potential and at high glucose (16.7 or 28 mmol/l), the endogenous V_M of INS-1 cells was above the I_{GABA} reversal potential. Therefore, GABA dose-dependently induced membrane depolarisation at a low glucose concentration, but hyperpolarisation at a high glucose concentration. Consistent with electrophysiological findings, insulin secretion assays demonstrated that at 2.8 mmol/l glucose, GABA increased insulin secretion in a dose-dependent fashion ($p < 0.05$, $n = 7$). This enhancement was blocked by bicuculline ($p < 0.05$, $n = 4$). In contrast, in the presence of 28 mmol/l glucose, GABA suppressed the secretion of insulin ($p < 0.05$, $n = 5$). *Conclusions/interpretation:* These findings indicate that activation of GABA_ARs in beta cells regulates insulin secretion in concert with changes in glucose levels.

Keywords Calcium influx · Gamma-aminobutyric acid · Glucose · INS-1 cells · Insulin secretion · Perforated patch clamp

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Abbreviations GABA_AR: A-type GABA receptor · [Ca²⁺]_i: intracellular Ca²⁺ concentration · $I-V$: current voltage · GABA: gamma-aminobutyric acid · V_M : membrane potential · I_{GABA}: GABA-induced transmembrane current

Introduction

Gamma-aminobutyric acid (GABA), which is synthesised from glutamic acid by the rate-limiting enzyme GAD, is a major inhibitory neurotransmitter in the mammalian central nervous system [1]. GABA induces rapid inhibition primarily by activating the A-type GABA receptor (GABA_AR) [2], a heteropentameric Cl⁻ ion channels complex [3]. In most cases, activation of GABA_AR in neurons results in membrane hyperpolarisation as a consequence of an inward Cl⁻ flux [3].

The concentrations of GABA and GAD and the density of GABA_AR in pancreatic islets are comparable with those

encountered in the central nervous system [4–8], suggesting that the GABA–GABA_AR system plays a role in modulating islet endocrine functions. In the pancreas, GABA is primarily produced by insulin-secreting beta cells [9–11] and probably by the glucagon-containing alpha cells [9, 12]. GABA_ARs are expressed in both beta and alpha cells [13–15], suggesting that GABA modulates pancreatic endocrine function in an autocrine and paracrine manner [16–18]. In pancreatic beta cells, GABA is associated with synaptic-like microvesicles that are distinct from the insulin-containing large dense-core vesicles [10, 11]. Release of GABA from beta cells appears to be regulated by nutrients [19] including glucose [19–21].

The effect of GABA on insulin secretion from the beta cells has also been controversial. For example, perfusion studies using rat and dog pancreas have shown that GABA inhibits arginine-stimulated insulin release [16, 21]. Other studies demonstrate that both GABA and GABA_AR agonists have no detectable effect on insulin secretion in perfused rat pancreas [22, 23]. However, GABA-induced increases in insulin release were observed in rat pancreas and isolated human insulinoma cells [13, 24, 25].

The direction of the GABA_AR-mediated Cl⁻ current depends on membrane potential (V_M), which, in pancreatic beta cells, is critically regulated by extracellular glucose [26, 27]. We hypothesise that the effect of GABA on insulin secretion from beta cells is dependent on the extracellular glucose concentration. Using perforated patch-clamp recordings and insulin secretion assays, we examined in insulin-secreting INS-1 cells the effects of GABA on V_M and insulin secretion in the presence of different levels of glucose. Our results indicate that, depending on the extracellular glucose levels, GABA can exert either excitatory or inhibitory effects on beta cells.

Materials and methods

Cell culture

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 µg/ml streptomycin sulphate, 55 mg/500 ml sodium pyruvate, 1.14 g/500 ml HEPES, and 1.7 µl/500 ml β-mercaptoethanol at 37°C in an atmosphere of humidified air (95%) and CO₂ (5%). In studies involving serum-starvation, serum was replaced by 0.1% BSA in RPMI 1640 without glucose.

RT-PCR

Total cellular RNA was extracted from confluent 10-cm plates of INS-1 cells using Trizol (Invitrogen Life Technologies, CA, USA) following the manufacturer's instructions. GABA_AR mRNA transcripts were detected using a one-step RT-PCR kit (Qiagen). Briefly, 100 ng total RNA were used in 25-µl one-step RT-PCR reactions

containing 0.4 mmol/l dNTPs and 0.6 µmol/l of each primer. The template was omitted for the negative control. Thermocycler conditions were 50°C for 30 min, 95°C for 15 min, and 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. This was followed by a 10-min extension at 72°C. The RT-PCR products were separated on a 1% agarose gel and visualised with ethidium bromide. The GABA_AR gene-specific primers used were as follows: α1 FP: cgggaagaagctatggaca; α1 RP: ccactcatagacaacttctgc; α2 FP: tctagacagacttctggatgg; α2 RP: agagtgcagaagcattg taagtc; α3 FP: cccagatattctgacgatag; α3 RP: tgagccatct gagtactt; β1 FP: cgactgctcaaaggatgatgac; β1 RP: agtga tagtctggtgatgccc; β2 FP: gctgtctgtctcagagtgt; β2 RP: gtgactgcattgtcatcgcc; β3 FP: acggtgcacaagctgttgaa; β3 RP: tcgagcagcagatgcatc; γ1 FP: gtccagatataggcgtgaga; γ1 RP: ctccagtacttaggatcagc; γ2 FP: gcattggtccagtgaatgct; γ2 RP: ggaatgtaggtctggtatggt; γ3 FP: acagcattggtcctgtgt ca; γ3 RP: caaactgatagagccgcat.

Immunoblotting

Cells were lysed in buffer containing 1% Triton X-100 and protease and phosphatase inhibitors, as previously described [28]. Proteins (50 µg) were separated by 10% SDS-PAGE and electrotransferred onto a nitrocellulose filter (0.2 µm; Bio-Rad Laboratories, Hercules, CA, USA). After probing with mouse anti-beta2/3 isoforms of GABA_AR IgG (1:1,000; Upstate Biotechnology, Lake Placid, NY, USA), the proteins were visualised with horseradish peroxidase-conjugated goat anti-mouse IgG (1:5,000; Upstate Biotechnology) using an electrochemiluminescence detection technique (Amersham, Piscataway, NJ, USA), as described previously [28].

Immunochemistry

Cells grown on glass coverslips in 12-well plates were fixed with paraformaldehyde (3.7% in PBS) and permeabilised with 0.2% Triton X-100. After incubation with 5% goat serum and 1% BSA (in PBS) for 60 min, cells were incubated with mouse antibodies against beta2/3 GABA_AR subunits (1:100; Upstate Biotechnology) overnight at 4°C. After washing, cells were incubated with Cy3-conjugated goat anti-mouse IgG (1:500, 30 min; Jackson Immuno-Research Laboratories, West Grove, PA, USA). Images were visualised using a Leica TCS 4D laser confocal fluorescence microscope.

Electrophysiology

Perforated patch recordings were performed at room temperature (23–25°C). One hour before recording, cells were bathed in the standard glucose-free extracellular solution containing (in mmol/l) 145 NaCl, 1.3 CaCl₂, 5.4 KCl, 25 HEPES, (pH 7.4). Recordings were performed using an Axopatch-1D amplifier (Axon Instruments, Foster

City, CA, USA). Electrodes (3–5 M Ω) were constructed from thin-walled glass (1.5 mm diameter; World Precision Instruments, Sarasota, FL, USA) using a two-stage puller (PP-830; Narishige, East Meadow, NY, USA). The standard intracellular solution for perforated patch recording consisted of (in mmol/l) 150 K-gluconate, 10 KCl, 10 KOH, 10 HEPES, 2 MgCl₂ and 1 CaCl₂. After addition of the pore-forming agent amphotericin B (Sigma-Aldrich Corp., Buchs, Switzerland) [27], the pH of the intracellular solution was adjusted to 7.30 (with KOH) and the osmolarity was corrected to a range of 310–315 mOsm.

Perforated recordings started under voltage-clamp mode. The effect of amphotericin B 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 30 M Ω within 5–15 min after the seal, and then stabilised for 45–60 min. All perforated patch recordings began when the serial resistance had attained values below 28 M Ω . To monitor a possible formation of whole-cell configuration, a testing voltage-ramp (a gradual voltage change from –80 to 60 mV in 1.5 s) was applied to the cell at the start of recording, and approximately every 10 min during the recording. With this testing protocol, a sigmoid-shaped current–voltage (I – V) curve was seen under stable perforated patch recordings, whereas a linear I – V relationship gradually appeared after whole-cell configuration. If a sudden change in the I – V relationship occurred, the recording was not used for the study. To measure the GABA_AR-channel activity in INS-1 cells, perforated patch recordings were carried out under voltage-clamp mode and cells were held at –60 mV unless otherwise indicated. Different concentrations of GABA (1.0–1000 μ mol/l) were focally applied to the recorded cells by means of a computer-controlled multi-barrelled perfusion system (SF-77B; Warner Instruments, Hamden, CT, USA). To investigate the effect of GABA on the excitability of INS-1

cells in different concentrations of glucose, changes in the V_M were evaluated via current-clamp recordings. The electrical signals were digitised, filtered (1 kHz), and acquired on-line using the program Clampex and analysed off-line by means of the program Clampfit (Axon Instruments).

Intracellular Ca²⁺ measurement

Intracellular Ca²⁺ concentration ($[Ca^{2+}]_i$) was measured using Fura 2-AM (Molecular Probes). Cells grown on coverslips (70–80% confluency) were loaded with Fura-2 AM (3 μ mol/l) in a medium containing (in mmol/l) 130 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 5 NaHCO₃ and 10 HEPES, pH 7.4 for 50 min at 37°C. Cells were washed and transferred to an open chamber equipped with a TC-324B Heater Controller (Warner Instruments) and perfused with the same medium at 1 ml/min (36–37°C) while the recordings were made using an Olympus BX51W1 fluorescent microscope. Cells were excited by dual excitation at 340/380 nm using a DeltaRam high-speed monochromator (Photon Technology International) and the emissions were detected at 510 nm by a band pass filter. The system was equipped with ImageMaster 3 software for control of the monochromator, the video camera, and collecting data.

Insulin secretion

Insulin concentrations were measured using a rat insulin RIA kit (Linco Research, St. Louis, MO, USA) as previous described [28]. INS-1 cells grown in 24-well plates to 85–90% confluency were rinsed twice and incubated with KRB (containing, in mmol/l, 115 NaCl, 5 KCl, 24 NaHCO₃, 2.5 CaCl₂, 1 MgCl₂, 10 HEPES, and 0.1% BSA) for

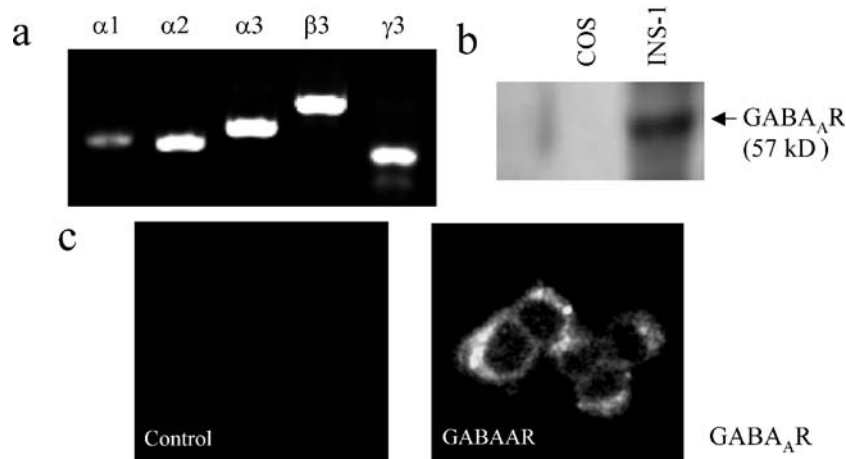


Fig. 1 Expression of GABA_A receptors in INS-1 cells. **a** RT-PCR to detect different GABA_AR subunit nucleotide sequences (See [Materials and methods](#)). Total RNA from INS-1 cells were used in one-step RT-PCR reactions using specific primers for the alpha_{1–3}, beta_{1–3}, and gamma_{1–3} subunits to detect expression of the different subunits. In the control reaction, the template was omitted.

b Western blot on INS-1 cell lysates (50- μ g protein) using a specific antibody against the beta_{2/3} subunits of the GABA_AR. COS cell lysates were used as control. **c** Immunocytochemistry of INS-1 cells using the antibody against beta_{2/3} subunits of the GABA_AR. COS cells were used in the control staining

60 min. Cells were incubated in KRB in the presence of various concentrations of glucose (dextrose; Merck KgaA, Darmstadt, Germany) and/or GABA (Sigma, St. Louis, MO, USA) as indicated. After 30 min of incubation, the media were collected and insulin levels measured by RIA using the rat insulin RIA kit according to the manufacturer's instructions. The insulin secretion was normalised to the cellular protein content. Protein was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, CA, USA).

Statistical analysis

Data are expressed as means \pm SEM. For insulin secretion assays, the number of samples and separate experiments are described in the figure legends. Statistical difference between groups was analysed by ANOVA followed by a Student–Newman–Keuls test to estimate the differences between each pair of groups when applicable. For electrophysiological analysis, data were compared with Student's unpaired or paired *t*-test where appropriate. A *p* value <0.05 was considered as significant. Statistical

analyses were performed using SigmaStat from Jandel Scientific Software (San Rafael, CA, USA).

Results

Expression of GABA_A receptor in INS-1 cells

RT-PCR experiments demonstrated that INS-1 cells express transcripts of GABA_AR subunits alpha 1, alpha 2, alpha 3, beta 3 and gamma 3 (Fig. 1a). It is known that in neurons, a functional GABA_AR is usually a pentamer composed of two alpha, two beta and one gamma receptor subunits [2]. Thus, INS-1 cells appear to possess the necessary subunits for a functional GABA_AR. Furthermore, Western blot analysis using a specific antibody directed against the beta2/3 subunits of GABA_AR detected the expression of GABA_AR proteins in INS-1 cells (Fig. 1b). Expression and membrane localisation of GABA_AR were also confirmed by immunostaining and confocal imaging studies (Fig. 1c). In addition, immunostaining of rat pancreas sections using anti-alpha1 and beta2/3 subunit antibodies showed that islet beta cells express alpha1 and beta2 or beta 3 subunits (data not shown).

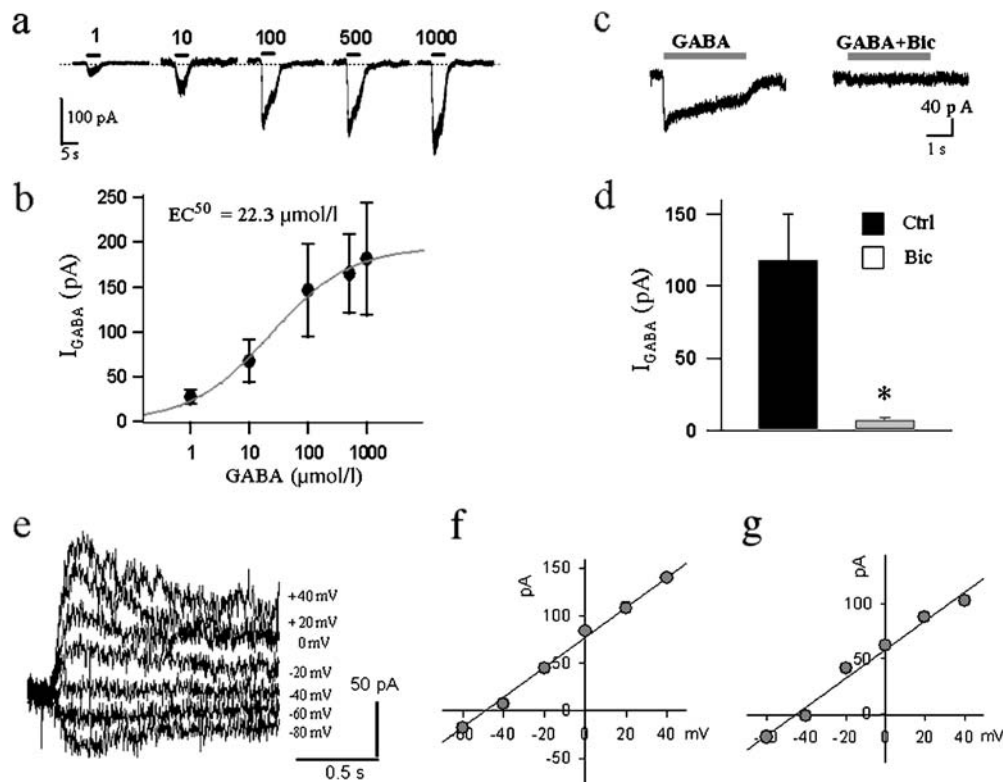


Fig. 2 Transmembrane current evoked by GABA. **a** GABA-evoked currents in INS-1 cells via activation of GABA_ARs. In a perforated voltage-clamp mode, when cells were voltage clamped at -60 mV, fast application of GABA (**b**) dose-dependently evoked inward currents ($n \geq 8$ cells at each testing concentration of GABA). **c** The effect of GABA_AR antagonist bicuculline (Bic, $50 \mu\text{mol/l}$) on GABA ($100 \mu\text{mol/l}$)-induced current. The peak amplitude (**d**) of GABA currents in the absence (Ctrl) and presence of bicuculline

was plotted (Ctrl: 117 ± 33 pA, $n=15$; Bic: 7 ± 2 pA, $n=4$; $p < 0.05$). **e** In the absence of glucose, GABA ($100 \mu\text{mol/l}$) evoked inward and outward currents in the same INS-1 cell when held at different membrane potentials. **f** The reversal potential for GABA currents in the absence of glucose was determined by analysing the current–voltage relationship (-42 ± 2.2 mV, $n=6$ cells). **g** In the presence of 16.7 -mmol/l glucose, GABA currents were reversed at -43 mV

GABA_ARs in INS-1 cells are functional

To investigate whether the GABA_ARs in INS-1 cells were functional, we applied different concentrations of GABA to the cells by means of a fast perfusion system, and measured transmembrane currents using voltage-clamp recording techniques (Fig. 2a). We found that when held at -60 mV, about 60% of cells generated visible inward currents in response to 1 $\mu\text{mol/l}$ GABA. Yet, currents were generated in all tested cells when GABA concentrations were higher than 3 $\mu\text{mol/l}$. The GABA-induced currents were concentration-dependent (Fig. 2a,b), with an EC_{50} of 22.3 $\mu\text{mol/l}$ (Fig. 2b). We noticed that at lower concentrations of GABA (<30 $\mu\text{mol/l}$), the amplitude of the GABA-evoked current (I_{GABA}) was stable (not shown). However, at higher concentrations (>100 $\mu\text{mol/l}$), I_{GABA} exhibited a rapid

onset, characterised by a peak, followed by a gradual decay to a steady state (Fig. 2c, left). In response to 100 $\mu\text{mol/l}$ GABA, the peak amplitude of the I_{GABA} in individual cells varied from 12 to 470 pA (Fig. 2d) (control I_{GABA} : 117 ± 33 pA, $n=15$). Notably, I_{GABA} could be reversibly suppressed by co-application of the competitive GABA_AR antagonist, bicuculline (50 $\mu\text{mol/l}$; bicuculline I_{GABA} : 7 ± 2 pA, $n=4$; $p < 0.05$) (Fig. 2d). These results demonstrate that the GABA_ARs in INS-1 cells are physiologically functional.

We further characterised the I - V relationship of GABA_A-R-gated channels in INS-1 cells. GABA (100 $\mu\text{mol/l}$) was applied to cells held at different V_{M} levels (-80 to 40 mV, see Fig. 2e) and the current was recorded. The plotted I - V curve indicated that in the absence of extracellular glucose, I_{GABA} reversed at -42 ± 2.2 mV (Fig. 2f, $n=6$). Glucose is one of the key physiological regulators of beta cell

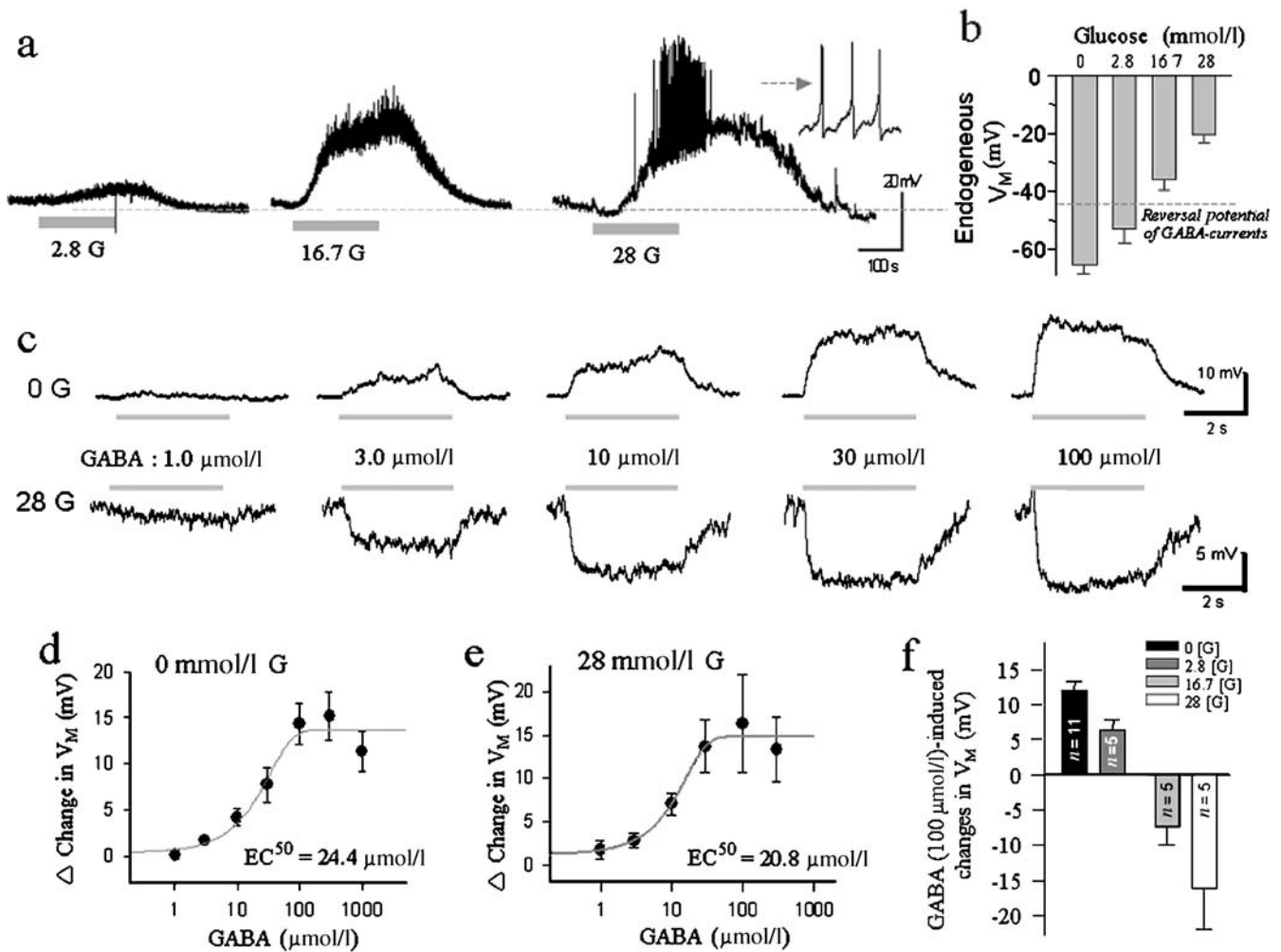


Fig. 3 Regulation of beta cell excitability by GABA in the presence of different concentrations of glucose. **a** Increase in glucose concentrations (G) from 0 mmol/l to 2.8 , 16.7 and 28 mmol/l, caused a dose-dependent depolarisation of the cell (traces at 2.8 , 16.7 and 28 G were from the same cell). Note that bursting of action potentials was generated in some cells in the presence of high glucose (**c**). **b** The membrane potentials (V_{M}) of INS-1 cells in the presence of various concentrations of glucose are summarised (at 0 mmol/l: -65 ± 3 mV, $n=12$; at 2.8 mmol/l: -53 ± 2 mV, $n=6$; at 16.7 mmol/l: -32 ± 2 mV, $n=7$; at 28 mmol/l: -22 ± 2 mV, $n=6$).

c Example traces illustrate the dose-dependent effect of GABA on V_{M} in the absence (0 G), and presence of 28 mmol/l (28 G) of glucose. The grey bars under or above each trace indicate the duration of GABA perfusion. **d**, **e** Summarised data reveal the concentration-dependency of GABA on V_{M} in 0 mmol/l and 28 mmol/l of glucose, respectively. **f** Plotted graph indicates the direction and the scope of GABA-induced changes in V_{M} at different glucose concentrations (0 mmol/l: 10 ± 1.2 mV, $n=11$; 2.8 mmol/l: 6.5 ± 0.7 mV, $n=5$; 16.7 mmol/l: -8.5 ± 5 mV, $n=5$; 28 mmol/l: -16.0 ± 5 mV, $n=5$).

functions. However, the reversal potential of I_{GABA} in INS-1 cells was not affected by addition of 16.7 mmol/l glucose to the extracellular solution (Fig. 2g), suggesting that glucose concentration does not affect this property of $GABA_A$ Rs, or the equilibrium potential of Cl^- in INS-1 cells.

GABA regulates the excitability of INS-1 cells differently under different glucose concentrations

Glucose concentrations dictate the ratio of intracellular ATP/ADP concentrations, which in turn regulate the activity of ATP-sensitive K^+ (KATP) channels, thereby controlling the V_M of beta cells [27, 29–31]. We tested the hypothesis that the level of glucose sets the instantaneous V_M , which in turn determines the effect of $GABA_A$ R activation on the excitability of beta cells. Using current-clamp recordings, we measured the change in V_M of INS-1 cells in response to increases of glucose concentration (0 to

2.8, 16.7 or 28 mmol/l, respectively, Fig. 3a). In the absence of glucose, V_M of INS-1 cells varied from -51 to -82 mV (Fig. 3b: -65 ± 3 mV, $n=12$). Increasing glucose concentrations depolarised the membrane of INS-1 cells in a concentration-dependent manner (Fig. 3a,b: V_M at 2.8 mmol/l glucose: -53 ± 2 mV; V_M at 16.7 mmol/l: -32 ± 2 mV; V_M at 28 mmol/l: -22 ± 2 mV, $n=6$). In some cases, bursts of action potentials were seen during high-glucose-induced depolarisation (Fig. 3a).

Next, we examined the effect of different concentrations of GABA on V_M at low and high concentrations of glucose (Fig. 3c). GABA (1–1,000 μ mol/l) induced membrane depolarisation as a function of dose in the absence of glucose, but caused membrane hyperpolarisation in the presence of 28 mmol/l glucose (Fig. 3c). The EC_{50} value of GABA on V_M at low glucose was similar to that at high glucose (EC_{50} at 0 mmol/l glucose: 24.4; EC_{50} at 28 mmol/l glucose: 20.8 μ mol/l, Fig. 3d,e), suggesting that glucose does not affect the affinity of GABA for $GABA_A$ Rs. We further examined GABA (100 μ mol/l)-induced changes in

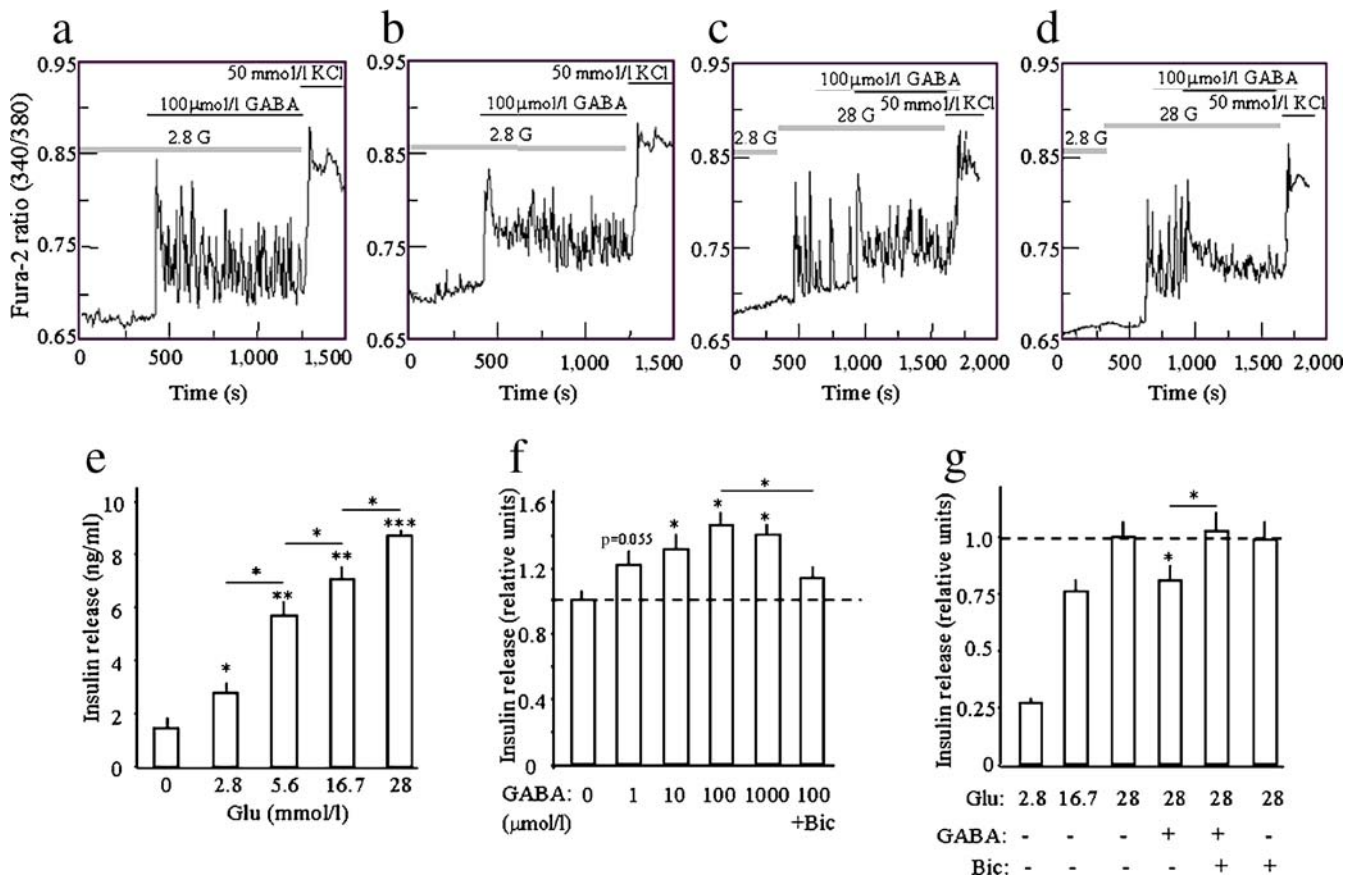


Fig. 4 Modulation of Ca^{2+} influx and insulin secretion by GABA in INS-1 cells. **a–d** Effect of GABA (100 μ mol/l) on $[Ca^{2+}]_i$ in INS-1 cells. Cells grown on coverslips were loaded with Fura-2 AM and transferred to a chamber and perfused at 1 ml/min (36–37°C) as described in **Materials and methods**. Horizontal lines show perfusion with corresponding reagents; the glucose (G) levels are outlined by the grey lines. The typical time-dependent $[Ca^{2+}]_i$ response from individual cells is shown and represents three independent experiments in which 30–50 cells were measured from each experiment at low glucose (**a**, **b**, 2.8 mmol/l) or high glucose (**c**,

d, 28 mmol/l). **e** Glucose-stimulated insulin secretion is concentration-dependent ($n=4$). Cells were incubated in KRB in the presence of various concentrations of glucose (Glu) as indicated. After 30 min of incubation, the media were collected and insulin levels were measured by RIA. **f** In the presence of 2.8 mmol/l glucose, insulin secretion was measured in the presence of different concentrations of GABA ($n=7$), with or without $GABA_A$ R antagonist bicuculline (Bic, 100 μ mol/l, $n=4$). **g** Effect of GABA (100 μ mol/l) and bicuculline (Bic, 100 μ mol/l) on insulin secretion at 28 mmol/l glucose (Glu, $n=4$). * $p<0.05$, ** $p<0.01$, *** $p<0.005$

V_M at 0, 2.8, 16.8 and 28 mmol/l glucose, and confirmed that GABA could up- and downregulate the V_M of beta cells in accordance with glucose concentrations (Fig. 3f, at 0 mmol/l glucose: depolarisation 12 ± 1.2 mV, $n=11$; at 2.8 mmol/l: depolarisation 6.5 ± 0.7 mV, $n=9$; at 16.8 mmol/l: hyperpolarisation -8.5 ± 5 mV, $n=9$; at 28 mmol/l: hyperpolarisation -16.0 ± 5 mV, $n=9$).

GABA increases the cytosolic calcium concentration of INS-1 cells

Using fluorescent probe, Fura 2-AM, we measured $[Ca^{2+}]_i$ of individual INS-1 cells in response to GABA treatment. Perfusion of GABA (100 μ mol/l) at low glucose (2.8 mmol/l) led to a significant increase of $[Ca^{2+}]_i$ (Fig. 4a,b: representative $[Ca^{2+}]_i$ of two individual cells). Presumably, this fact reflects depolarisation of the cells by GABA, because membrane depolarisation induced by increasing extracellular KCl (to 50 mmol/l) also drastically elevated $[Ca^{2+}]_i$ of the INS-1 cells. In all cells tested, the increase of $[Ca^{2+}]_i$ during GABA treatment displayed oscillatory behaviour (Fig. 4a,b, $n=3$; 30–50 cells were measured from each experiment). Perfusion of 28 mmol/l glucose led to a drastic increase in cytosolic Ca^{2+} oscillation (Fig. 4c,d). In this case, application of GABA (100 μ mol/l) suppressed the amplitude of Ca^{2+} oscillation spikes (Fig. 4c,d). Nevertheless, an increase in the frequency of Ca^{2+} oscillation was observed in some cells (Fig. 4c).

GABA-modulated insulin secretion in INS-1 cells is dependent on glucose concentration

Next, we determined whether GABA affects insulin secretion in INS-1 cells using insulin secretion assays. Glucose stimulated insulin secretion from INS-1 cells in a dose-dependent manner (Fig. 4e). Consistent with the electrophysiological data, under low glucose conditions (2.8 mmol/l), GABA (1–1,000 μ mol/l) steadily increased insulin secretion (Fig. 4f, $p < 0.05$, $n=7$). This effect of GABA was significantly diminished by the GABA_AR antagonist bicuculline (Fig. 4f, $n=4$). In contrast, under high glucose conditions (28 mmol/l), GABA significantly suppressed insulin secretion (Fig. 4g, $p < 0.05$, $n=5$). This suppression was also significantly attenuated by co-incubation of the cells with bicuculline (Fig. 4g, $p < 0.05$, $n=4$). Bicuculline, by itself, had no significant effect on insulin secretion under low and high glucose conditions (Fig. 4f,g, $p > 0.05$, $n=4$). In contrast, the GABA_BR agonist, baclofen had no significant effect on GABA-modulated insulin secretion from INS-1 cells at either low (2.8 mmol/l) or high (28 mmol/l) glucose (2.8 mmol/l: control vs. baclofen = 1.0 ± 0.1 vs. 0.98 ± 0.09 ; 28 mmol/l: control vs. baclofen = 1 ± 0.16 vs. 1.12 ± 0.19 ; $p > 0.05$, $n=3$). These results indicate that activation of GABA_AR up- and downregulates insulin secretion depending on the extracellular concentration of glucose.

Discussion

Previous studies [4–8] suggest that the pancreatic GABA–GABA_AR system plays a role in modulating islet endocrine functions. However, the reported effects of GABA on insulin secretion from the beta cells have been controversial. In this study we demonstrated a mechanism by which GABA modulates insulin secretion from beta cells in concert with changes in glucose concentration.

We found that GABA_ARs are expressed in the widely used beta cell line INS-1 cells (Fig. 1), which is consistent with previously reported studies in isolated human insulinoma cells [13] and rat pancreatic beta cells [15]. Since GABA is localised mainly within the pancreatic beta cells [32], identification of GABA_ARs in the beta cells suggests that the GABA–GABA_AR system may serve as both an autocrine and paracrine modulator in the islets. We also demonstrated that, dependent on dose, perfusion of GABA induced transmembrane currents in INS-1 cells, and that the GABA-induced current was sensitive to the GABA_AR antagonist, bicuculline (Fig. 2a,c). These results indicate that GABA_ARs in INS-1 cells are functional.

It is well known that by modulating the KATP conductance, glucose concentration critically sets the instantaneous V_M of beta cells [27]. We confirmed that with increasing glucose concentrations, from 0 to 28 mmol/l, the V_M of INS-1 cells changed from approximately -65 mV to approximately -22 mV. On the other hand, we found that changing glucose concentrations did not affect the reversal potential of GABA_AR-mediated currents, which was at about -42 mV in INS-1 cells. Therefore, activation of GABA_AR could potentially up- and downregulate the excitability of the cell, in accordance with changes in glucose concentrations. Indeed, our results demonstrated that while the cell membrane hyperpolarised in the presence of low glucose, application of GABA positively shifted the V_M . In contrast, under conditions of high glucose, GABA negatively shifted the elevated V_M . These data suggest that GABA, in concert with glucose, plays a role in regulation of excitability of the beta cell, thus regulating insulin secretion. This notion was strongly supported by the findings that GABA elevated the $[Ca^{2+}]_i$ oscillations at low glucose (Fig. 4a), but suppressed the amplitude of high-glucose-induced Ca^{2+} -oscillation spikes (Fig. 4c).

Our insulin RIA results further confirmed that GABA stimulated insulin secretion at low (2.8 mmol/l) glucose, but inhibited insulin secretion at high (28 mmol/l) glucose. The bi-directional regulation of insulin secretion in beta cells by GABA at different glucose levels implies an important role for GABA in modulating islet hormonal secretion. GABA, by modulating insulin secretion from beta cells in concert with changes in glucose concentration, provides a unique mechanism for pancreatic endocrine regulation. Notably, in the presence of low glucose (2.8 mmol/l), GABA caused a moderate but significant increase in insulin secretion (~ 20 – 40% , equivalent to ~ 0.3 – 0.4 nmol/l). Intracellular Ca^{2+} measurements sug-

gested that GABA-modulated insulin secretion in INS-1 cells may be via a mechanism involving Ca^{2+} movement.

Our findings in this study suggest that the GABA system may function as a negative feedback regulating mechanism in the islets. Under low glucose conditions, GABA release from beta cells increases [33, 34], which in turn may enhance insulin secretion. It has been demonstrated that insulin is a physiological inhibitor of glucagon release within islets [35] under in vitro [36, 37] and in vivo [38] conditions. Studies on islet microvasculature have shown that the alpha cells lie downstream from the beta cells, thus insulin released from beta cells can immediately act on alpha cells [39]. Therefore, physiologically, a modest change in insulin concentrations in the islet could have a significant impact on glucagon secretion from alpha cells. For instance, when hypoglycaemia occurs, GABA-stimulated insulin may act on the alpha cells to prevent the potential hyperglycaemia resulting from excessive glucagon secretion, because glucagon is a prominent facilitator of glucose production [40, 41]. Likewise, during hyperglycaemia, GABA depresses insulin secretion thus preventing a potential hypoglycaemia resulting from insulin 'overshooting'.

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References

- Gottlieb DI (1988) GABAergic neurons. *Sc Am* 258:82–89
- Kittler JT, McAinsh K, Moss SJ (2002) Mechanisms of GABA_A receptor assembly and trafficking: implications for the modulation of inhibitory neurotransmission. *Mol Neurobiol* 26:251–268
- Kittler JT, Moss SJ (2003) Modulation of GABA_A receptor activity by phosphorylation and receptor trafficking: implications for the efficacy of synaptic inhibition. *Curr Opin Neurobiol* 13:341–347
- Briel G, Gylfe E, Hellman B, Neuuhoff V (1972) Microdetermination of free amino acids in pancreatic islets isolated from obese-hyperglycemic mice. *Acta Physiol Scand* 84:247–253
- Garry DJ, Coulter HD, McIntee TJ, Wu JY, Sorenson RL (1987) Immunoreactive GABA transaminase within the pancreatic islet is localized in mitochondria of the B-cell. *J Histochem Cytochem* 35:831–836
- Michalik M, Erecinska M (1992) GABA in pancreatic islets: metabolism and function. *Biochem Pharmacol* 44:1–9
- Okada Y, Taniguchi H, Schimada C (1976) High concentration of GABA and high glutamate decarboxylase activity in rat pancreatic islets and human insulinoma. *Science* 194:620–622
- Michalik M, Nelson J, Erecinska M (1993) GABA production in rat islets of Langerhans. *Diabetes* 42:1506–1513
- Chessler SD, Simonson WT, Sweet IR, Hammerle LP (2002) Expression of the vesicular inhibitory amino acid transporter in pancreatic islet cells: distribution of the transporter within rat islets. *Diabetes* 51:1763–1771
- Thomas-Reetz A, Hell JW, During MJ, Walch-Solimena C, Jahn R, De Camilli P (1993) A gamma-aminobutyric acid transporter driven by a proton pump is present in synaptic-like microvesicles of pancreatic beta cells. *Proc Natl Acad Sci USA* 90:5317–5321
- Sorenson RL, Garry DG, Brelje TC (1991) Structural and functional considerations of GABA in islets of Langerhans. Beta-cells and nerves. *Diabetes* 40:1365–1374
- Gilon P, Campistron G, Geffard M, Remacle C (1988) Immunocytochemical localisation of GABA in endocrine cells of the rat entero-pancreatic system. *Biol Cell* 62:265–273
- Glassmeier G, Hopfner M, Buhr H et al (1998) Expression of functional GABA_A receptors in isolated human insulinoma cells. *Ann NY Acad Sci* 859:241–248
- Rorsman P, Berggren PO, Bokvist K et al (1989) Glucose-inhibition of glucagon secretion involves activation of GABA_A -receptor chloride channels. *Nature* 341:233–236
- Von Blankenfeld G, Turner J, Ahnert-Hilger G et al (1995) Expression of functional GABA_A receptors in neuroendocrine gastropancreatic cells. *Pflugers Arch* 430:381–388
- Gu XH, Kurose T, Kato S et al (1993) Suppressive effect of GABA on insulin secretion from the pancreatic beta-cells in the rat. *Life Sci* 52:687–694
- Satin LS, Kinard TA (1998) Neurotransmitters and their receptors in the islets of Langerhans of the pancreas: what messages do acetylcholine, glutamate, and GABA transmit? *Endocrine* 8:213–223
- Shi Y, Kanaani J, Menard-Rose V et al (2000) Increased expression of GAD65 and GABA in pancreatic beta-cells impairs first-phase insulin secretion. *Am J Physiol Endocrinol Metab* 279:E684–E694
- Smismans A, Schuit F, Pipeleers D (1997) Nutrient regulation of gamma-aminobutyric acid release from islet beta cells. *Diabetologia* 40:1411–1415
- Winnock F, Ling Z, De Proft R et al (2002) Correlation between GABA release from rat islet beta-cells and their metabolic state. *Am J Physiol Endocrinol Metab* 282:E937–E942
- Kawai, Unger RH (1983) Effects of gamma-aminobutyric acid on insulin, glucagon, and somatostatin release from isolated perfused dog pancreas. *Endocrinology* 113:111–113
- Gilon P, Bertrand G, Loubatieres-Mariani MM, Remacle C, Henquin JC (1991) The influence of gamma-aminobutyric acid on hormone release by the mouse and rat endocrine pancreas. *Endocrinology* 129:2521–2529
- Robbins MS, Grouse LH, Sorenson RL, Elde RP (1981) Effect of muscimol on glucose-stimulated somatostatin and insulin release from the isolated, perfused rat pancreas. *Diabetes* 30:168–171
- Adeghate E, Ponery AS (2002) GABA in the endocrine pancreas: cellular localization and function in normal and diabetic rats. *Tissue Cell* 34:1–6
- Gomez R, Asnis N, Tannhauser SL, Barros HM (1999) GABA agonists differentially modify blood glucose levels of diabetic rats. *Jpn J Pharmacol* 80:327–331
- Maechler P, Wollheim CB (2001) Mitochondrial function in normal and diabetic beta-cells. *Nature* 414:807–812
- Merglen A, Theander S, Rubi B, Chaffard G, Wollheim CB, Maechler P (2004) Glucose sensitivity and metabolism-secretion coupling studied during two-year continuous culture in INS-1E insulinoma cells. *Endocrinology* 145:667–678
- Wang Q, Li L, Xu E, Wong V, Rhodes C, Brubaker PL (2004) Glucagon-like peptide-1 regulates proliferation and apoptosis via activation of protein kinase B in pancreatic INS-1 beta cells. *Diabetologia* 47:478–487
- Ashcroft FM, Ashcroft SJ, Harrison DE (1988) Properties of single potassium channels modulated by glucose in rat pancreatic beta-cells. *J Physiol* 400:501–527

30. Theler JM, Mollard P, Guerineau N et al (1992) Video imaging of cytosolic Ca^{2+} in pancreatic beta-cells stimulated by glucose, carbachol, and ATP. *J Biol Chem* 267:18110–18117
31. Ashcroft FM, Rorsman P (1989) Electrophysiology of the pancreatic beta-cell. *Prog Biophys Mol Biol* 54:87–143
32. Reetz A, Solimena M, Matteoli M, Folli F, Takei K, De Camilli P (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
33. Hayashi M, Yamada H, Uehara S et al (2003) Secretory granule-mediated co-secretion of L-glutamate and glucagon triggers glutamatergic signal transmission in islets of Langerhans. *J Biol Chem* 278:1966–1974
34. Uehara S, Muroyama A, Echigo N et al (2004) Metabotropic glutamate receptor type 4 is involved in autoinhibitory cascade for glucagon secretion by alpha-cells of islet of Langerhans. *Diabetes* 53:998–1006
35. Maruyama H, Hisatomi A, Orci L, Grodsky GM, Unger RH (1984) Insulin within islets is a physiologic glucagon release inhibitor. *J Clin Invest* 74:2296–2299
36. Kaneko K, Shirotani T, Araki E et al (1999) Insulin inhibits glucagon secretion by the activation of PI3-kinase in In-R1-G9 cells. *Diabetes Res Clin Pract* 44:83–92
37. Kisanuki K, Kishikawa H, Araki E et al (1995) Expression of insulin receptor on clonal pancreatic alpha cells and its possible role for insulin-stimulated negative regulation of glucagon secretion. *Diabetologia* 38:422–429
38. Greenbaum CJ, Havel PJ, Taborsky GJ Jr, Klaff LJ (1991) Intra-islet insulin permits glucose to directly suppress pancreatic A cell function. *J Clin Invest* 88:767–773
39. Bonner-Weir S, Orci L (1982) New perspectives on the microvasculature of the islets of Langerhans in the rat. *Diabetes* 31:883–889
40. Raskin P, Unger RH (1978) Hyperglucagonemia and its suppression. Importance in the metabolic control of diabetes. *N Engl J Med* 299:433–436
41. Johnson DG, Goebel CU, Hruby VJ, Bregman MD, Trivedi D (1982) Hyperglycemia of diabetic rats decreased by a glucagon receptor antagonist. *Science* 215:1115–1116