Long-term inhibition of protein tyrosine kinase impairs electrophysiologic activity and a rapid component of exocytosis in pancreatic β-cells

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Abstract

Dysfunction of pancreatic β-cells is a fundamental feature in the pathogenesis of type 2 diabetes. As insulin receptor signaling occurs via protein tyrosine kinase (PTK), we investigated the role of PTK activity in the etiology of β-cell dysfunction by inhibiting PTK activity in primary cultured mouse pancreatic β-cells and INS-1 cells with genistein treatment over 24 h. Electrophysiologic recordings showed genistein treatment significantly attenuated ATP-sensitive K+ (KATP) and voltage-dependent Ca2+ currents, and depolarized the resting membrane potential in primary β-cells. When stimulated by high glucose, genistein-treated β-cells exhibited a time delay of both depolarization and Ca2+ influx, and were unable to fire action potentials, as well as displaying a reduced level of Ca2+ influx and a loss of Ca2+ oscillations. Semiquantitative PCR analysis revealed decreased expression of KATP and L-type Ca2+ channel mRNA in genistein-treated islets. PTK inhibition also significantly reduced the rapid component of secretory vesicle exocytosis, as indicated by membrane capacitance measurements, and this is likely to be due to the reduced Ca2+ current amplitude in these cells. These results illustrate that compromised PTK activity contributes to pancreatic β-cell dysfunction and may be involved in the etiology of type 2 diabetes.

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Introduction

Normal glucose homeostasis requires finely controlled orchestration of insulin secretion in response to subtle changes in blood glucose levels. Glucose-induced insulin secretion occurs when pancreatic β-cells utilize glucose to generate ATP from ADP. The resulting increase in cytoplasmic ATP/ADP ratio closes ATP-sensitive K+ channels (KATP channels), causing depolarization of the plasma membrane, which activates voltage-dependent Ca2+ channels. This results in an elevation of the intracellular Ca2+ concentration ([Ca2+]i) which triggers insulin secretion (Pretkí et al. 1997, Rorsman 1997). In type 2 diabetes, pancreatic β-cells exhibit atypical ion channel activity and an abnormal pattern of insulin secretion (Ashcroft & Rorsman 2004).

Hyperglycemia and hyperlipidemia are two major factors thought to contribute to the dysfunction of β-cells in type 2 diabetes. Clinical studies in diabetic patients and studies in Zucker diabetic fatty rats have shown that β-cell dysfunction takes place before the development of hyperglycemia (Tokuyama et al. 1995, Weyer et al. 1999, Matthews 2001, van Haeften 2002). The toxic effect of free fatty acids (FFAs) on β-cells is dependent on hyperglycemia in type 2 diabetes (Jacqueminet et al. 2000, Poitout & Robertson 2002), and β-cell dysfunction in this disease is able to occur in the absence of any increase in plasma FFAs (Jonas et al. 1999, de Souza et al. 2000). Therefore, it seems possible that neither ‘glucotoxicity’ nor ‘lipotoxicity’ may fully explain the onset of β-cell dysfunction in type 2 diabetes.

Insulin resistance in fat, muscle and liver cells is another major phenomenon in the development of type 2 diabetes, and insulin resistance in the β-cell may also be a key link in fully understanding the occurrence of β-cell dysfunction in this disease. Although the role of insulin action on β-cells remains controversial, insulin has been suggested to regulate its own secretion and the expression of several important genes in β-cells (Elahi et al. 1982, Ammon et al. 1991, Borge et al. 2002). Moreover, tissue-specific knockout of the insulin receptor in mouse pancreatic β-cells produces defects in insulin secretion similar to that in type 2 diabetes (Kulkarni et al. 1999a), strongly supporting the notion that insulin resistance in these cells could lead to β-cell dysfunction.

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The insulin signaling pathway in pancreatic β-cells occurs when insulin binds to insulin receptors, which possess protein tyrosine kinase (PTK) activity. Crucial mechanisms such as β-cell growth, differentiation and survival are regulated via PTK signaling pathways (Rhodes & White 2002). The acute application of genistein, a selective PTK inhibitor (Akiyama et al. 1987), reduces glucose induced Ca\(^{2+}\) influx in mouse β-cells by multiple pathways but paradoxically stimulates insulin secretion (Jonas et al. 1995). In this study, we have chronically exposed pancreatic β-cells to genistein in order to elucidate the effect of PTK inhibition on normal β-cell function. We have concentrated on evaluating the effect of this PTK inhibition on several parameters of β-cell function, including electrical activity, [Ca\(^{2+}\)]\(_i\) response to high glucose and secretory capacity.

Materials and methods

Preparation and culture of mouse islets, β-cells and INS-1 cell line

Pancreatic islets were isolated from 8–10 week-old C57BL/6 male mice. The mice were killed by decapitation, as approved by the Monash Medical Centre Animal Care Committee. The pancreas was inflated by injecting Hank’s solution containing 1 mg/ml collagenase (type V; Sigma, Sigma-Aldrich, St. Louis, MO, USA) through the bile duct into the pancreas. The pancreas was then isolated, removed and digested at 37°C for 20 min. The islets were separated by Histopaque-1077 (Sigma) centrifugation and selected by hand under a stereomicroscope (Li & Shapiro 1995). Islets were cultured on 35 mm plastic dishes and harvested for RNA extraction after treatment with or without 0·1 mM genistein for 24 h. For single-cell recording, the islets were dispersed into single cells by digestion with 1 mg/ml dispase (Sigma) (Josefsen et al. 1996). Cells were plated into 35 mm plastic dishes for electrophysiologic recording and onto glass cover slips coated with 0·01% poly-L-lysine for measuring [Ca\(^{2+}\)]\(_i\). For experiments performed with clonal insulin-releasing INS-1 cells, these cells were split weekly with 0·1% trypsin EDTA and replated on 35 mm plastic dishes for at least 2 days before experimental use. All cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO\(_2\). Cells were treated with or without 0·1 mM genistein for 24 h. Cells which were not treated with genistein will be referred to as WT cells, while those exposed to the PTK inhibitor will be referred to as genistein-treated cells. Culture medium with or without addition of 17β-estradiol (1 µg/ml) did not have any effect on either recorded Ca\(^{2+}\) or K\(^+\) currents in cultured mouse β-cells (data not shown), a result which excludes the influence of estrogen receptor activity by genistein treatment in the present experiments. Genistin, a compound structurally similar to genistein, was tested in the system without any effect on either Ca\(^{2+}\) or K\(^+\) currents in mouse β-cells (data not shown). Experiments were performed 2–5 days after plating.

Electrophysiologic recording

Recordings were made with the perforated whole-cell patch clamp configuration. Electrodes were pulled by a Sutter P-87 microelectrode puller from borosilicate micropipettes (Clark Electromedical Instruments, Pangbourne, Reading, UK) with an input resistance of 3–5 MΩ. Recordings were performed with the Axopatch 200A amplifier (Axon Instruments, Union City, CA, USA) for primary mouse β-cells and an EPC-9 amplifier in conjunction with the Pulse software (version 8·53; HEKA, Lambrecht, Germany) for INS-1 cells. The bath solution for Ca\(^{2+}\) current recordings from primary β-cells contained (mM): 140 NaCl, 4·7 KCl, 2·6 CaCl\(_2\), 1·2 MgCl\(_2\), 2 NaHCO\(_3\), 1·2 KH\(_2\)PO\(_4\), 5 glucose and 5 HEPES (pH 7·4). The pipette solution for these recordings contained (mM): 76 Cs\(_2\)SO\(_4\), 58 CsCl, 5 NaCl, 8 MgSO\(_4\) and 20 HEPES (pH 7·3). The bath solution for K\(^+\) current and membrane potential recordings contained (mM): 140 NaCl, 4·7 KCl, 2·6 CaCl\(_2\), 1·2 MgCl\(_2\), 2 NaHCO\(_3\), 1·2 KH\(_2\)PO\(_4\), 2 glucose and 5 HEPES (pH 7·4). The pipette solution for these recordings contained (mM): 76 K\(_2\)SO\(_4\), 58 KCl, 5 NaCl, 8 MgSO\(_4\) and 20 HEPES (pH 7·3). For capacitance and Ca\(^{2+}\) current recordings from INS-1 cells, the pipette solution contained (mM): 50 CsCl, 75 Cs\(_2\)SO\(_4\), 10 NaCl, 3 MgSO\(_4\) and 10 HEPES (pH 7·3). The external solution for these recordings consisted of (mM): 20 TEA-Cl, 118 NaCl, 5 KCl, 5 CaCl\(_2\), 0·5 MgCl\(_2\), 5 HEPES and 3 glucose (pH=7·4). Na\(^+\) current analysis from INS-1 cells was also carried out with these same solutions, as the Na\(^+\) and Ca\(^{2+}\) currents are clearly temporally separated, removing the need to utilize specific Ca\(^{2+}\) channel antagonists to measure these Na\(^+\) currents. Membrane perforation was achieved by use of amphotericin B (0·24 mg/ml) for primary β-cells or nystatin (0·2 mg/ml) for INS-1 cells. After formation of a high-resistance seal, cells were held at –70 mV until a stable series resistance lower than 30 MΩ was achieved. Whole-cell capacitance and series resistance were compensated before experiments. Exocytosis was monitored as changes in cell capacitance, using the sine+DC mode of the lock-in amplifier (40 mV peak-to-peak amplitude wave at 1 kHz) included in the Pulse software suite (Pulse Software Inc., Elizabeth, NJ, USA). Secretion was elicited by trains of 10, 300-ms depolarizations (1 Hz stimulation frequency) from –70 to
0 mV. During all patch-clamp experiments, cells were continuously superfused with the extracellular medium at a rate of ~2 ml/min. All electrophysiologic measurements were performed at room temperature except for capacitance experiments, which were performed at 32–34 °C.

Measurement of [Ca2+]

Primary mouse β-cells were loaded with 1 µM Fura-2/AM in RPMI-1640 medium for 30 min at 37 °C. Cells were subsequently rinsed with bath solution and kept for at least 20 min to allow for full de-esterification of the dye and to equilibrate in the cells. The bath solution used for [Ca2+]i measurement was identical to that used for membrane potential recording. Fluorescent measurements were performed with a dual excitation microfluorescence system and the data recorded with the Axoscope 8.2 program (Axon Instruments). Fura-2 was excited at 340 and 380 nm, with the emitted fluorescence detected at 510 nm. Changes in [Ca2+]i were calculated as the ratio of the fluorescence measured when cells were excited at 340 and 380 nm to give F340 nm/F380 nm. These experiments were carried out at room temperature.

Semiquantitative RT-PCR

Total RNA from mouse pancreatic islets was extracted with Trizol reagent (GIBCO BRL, Gaithersburg, MD, USA). The procedures followed were according to the reagent instructions (Roche). Briefly, RNA was dissolved in RNase-free water, and RNA quantity and quality were evaluated by measuring optical density at 260 and 280 nm; results were further confirmed by RNA electrophoresis. All RNA samples were adjusted to 0.25 µg/µl. Reverse transcription (RT) was performed in a 20 µl solution containing 1 µg total RNA plus reverse transcriptase, random primers, and dNTP. After incubation for 2 h at 46 °C, the reaction mixture was heated to 95 °C for 5 min to inactivate the RT. cDNA was then used for PCR. Primers for KATP channel subunit Kir6.2, Ca2+ channels subunit α1C and internal control 18S were designed by primer design software or as cited in previous reports (Gu et al. 2001, Nie et al. 2003). The conditions for each pair of primers, including annealing temperature and cycle numbers, were optimized in pilot experiments. The GenBank numbers, product sizes, sequences, annealing temperatures and cycle numbers for each pair of primers are listed in Table 1. The reaction mixture for PCR was composed of 4 µl 10× PCR buffer, 8 µl dNTP (100 µM), 1 µl forward primer (15 pmol/µl), 1 µl reverse primer (15 pmol/µl), 23.5 µl H2O, 0.5 µl Taq polymerase and 2 µl cDNA. The reaction mixture was denatured for 5 min at 94 °C and then subjected to cycles of denaturing at 94 °C for 30 s, annealing for 30 s and extending at 72 °C for 30 s. At the end of the last cycle, the extension time was changed to 10 min. PCR products were checked by electrophoresis on a 1.4% agarose gel stained with ethidium bromide. Gels were imaged under UV light and transferred to computer files. Bands were quantified by Quantity One software (Life Science, New York, NY, USA). Relative levels of amplified mRNA were normalized to those of 18S signals (internal control).

Data analysis

Results are presented as mean ± s.e.m. One-way ANOVA was used to compare differences between treatment groups and controls, and Dunnett’s post hoc test was used when appropriate. P<0.05 was set as the limit for statistical significance. All experiments conformed to the National Health and Medical Research Council (Australia) ethics code of practice.

Results

Genistein treatment decreases membrane potential and KATP currents in primary β-cells

For patch clamp recordings, cells were held at −70 mV, and K+ current was measured by ramping the voltage
from –120 to 20 mV over 500 ms (Fig. 1A and B). Tolbutamide (0·1 mM) reduced K+ current amplitude in both treatment groups, consistent with the presence of KATP currents. We subtracted the ramp current during tolbutamide exposure from the control current to give total KATP current (Fig. 1 inset). The KATP current is larger in WT cells than genistein-treated cells, indicating downregulation of functional KATP currents in the treated cells. The voltage at which these currents reverse is close to the expected equilibrium potential for K+ ions under our experimental conditions, further confirming that this is indeed a K+-selective ion channel that we have isolated by this method. The mean effect of tolbutamide was indeed significantly reduced in genistein-treated cells, confirming a downregulation of KATP currents due to PTK inhibition (Fig. 1C). Total K+ current density was reduced from –33·3 ± 6·3 pA/pF at –120 mV in WT cells to –15·4 ± 3·9 pA/pF in genistein-treated β-cells (Fig. 1C) (n=6, P<0·01). These values were reduced by tolbutamide to –10·2 ± 1·8 pA/pF in WT cells and –9·5 ± 0·3 pA/pF in genistein-treated cells (P<0·05).

The resting membrane potential of WT β-cells measured in current clamp mode was –58·9 ± 0·4 mV, decreasing to –75·2 ± 2·2 mV in the presence of the KATP channel agonist, diazoxide (n=8, P<0·05) compared with –46·5 ± 1·7 mV, decreasing to –54·0 ± 2·8 mV with 0·2 mM diazoxide in genistein-treated β-cells (n=6, P<0·05). Membrane potentials were significantly depolarized in genistein-treated β-cells, and the effect of diazoxide in genistein-treated cells was also significantly lower than in WT cells (Fig. 1D) (P<0·05), again indicating KATP channel downregulation by PTK inhibition.

PTK inhibition reduces Ca2+ current size in primary β-cells and INS-1 cells

Cells were held at –70 mV and Ca2+ currents evoked with depolarizing pulses in 10 mV steps from –50 mV to 60 mV (Fig. 2A). These Ca2+ currents were dramatically attenuated in genistein-treated β-cells (Fig. 2B). The current–voltage relationship of these Ca2+ currents shows decreased current across many testing voltages in

![Figure 1](https://example.com/figure1.png)
genistein-treated primary β-cells (Fig. 2C). The average maximum Ca²⁺ current density in WT primary β-cells was −13.8 ± 1.5 pA/pF (n=8), significantly greater than −7.0 ± 1.5 pA/pF in genistein-treated β-cells (n=5, P<0.01). The effect of PTK inhibition on Ca²⁺ currents was similar in INS-1 cells (Fig. 2D), as the peak current density in WT INS-1 cells was significantly greater than in genistein-treated cells (−12.9 ± 0.7 pA/pF (n=19) vs −9.4 ± 1.4 pA/pF (n=11) respectively; P<0.05).

**Action potential firing in response to high glucose is lost in genistein-treated cells**

When stimulated by 15 mM glucose, WT primary β-cells quickly depolarized from their resting membrane potential and fired action potentials of approximately 80-ms duration (Fig. 3A). The frequency of action potential firing was 2–4 Hz in the cells recorded in this study. While the membrane potential eventually reached similar depolarized levels in genistein-treated β-cells, these cells rarely fired any action potentials, except for small action potentials recorded in two cells at the very beginning of the depolarization, and these continued over only a very short period (Fig. 3B). Genistein-treated cells displayed an increased latent period before depolarization also (89 ± 13 s in WT cells; 264 ± 65 s in genistein-treated cells; P<0.01, n=6).

**Glucose-stimulated Ca²⁺ influx is altered by PTK inhibition in primary β-cells**

In Fura-2 loaded primary β-cells, no significant difference in basal fluorescence levels was observed between WT and genistein-treated cells. After stimulation with 15 mM glucose, WT β-cells exhibited a faster and greater [Ca²⁺]ᵢ increase (Fig. 4A) than that in genistein-treated cells (Fig. 4B). The latent periods before an increase in [Ca²⁺]ᵢ was prolonged from 165 ± 17 s in WT β-cells to 430 ± 147 s in genistein-treated cells (P<0.001, n=4). This is in line with the increase in time taken until these genistein-treated cells were fully depolarized by 15 mM glucose (Fig. 3B). The magnitude of [Ca²⁺]ᵢ increase was also smaller in genistein-treated, primary β-cells, the peak ratio of F340/F380 nm being 2.55 ± 0.05-fold greater than the basal level in WT β-cells (P<0.05, n=4), but only 1.82 ± 0.08-fold higher (P<0.05, n=4) than the basal level in genistein-treated β-cells (P<0.05). Under continuous stimulation by high glucose, WT β-cells exhibited Ca²⁺ oscillations, whereas genistein-treated β-cells exhibited no such oscillations, producing only single increases in [Ca²⁺]ᵢ in response to high glucose.
Genistein treatment decreases the expression of K\textsubscript{ATP} and Ca\textsuperscript{2+} channel genes

Genistein-mediated decreases in K\textsubscript{ATP} and Ca\textsuperscript{2+} currents could be due to the inhibition of channel gene expression. K\textsubscript{ATP} channel subunit Kir6·2 and the Ca\textsuperscript{2+} channel subunit \(\alpha_{1C}\) were therefore quantified by semiquantitative RT-PCR (Fig. 5A). The reasoning behind choosing these two specific genes for quantification is provided in the Discussion section. Semiquantitative RT-PCR results suggest that the mRNA expression of these two channels is downregulated in primary \(\beta\)-cells by genistein treatment (Fig. 5B) (\(n=6\)), with mRNA levels for Kir6·2 and \(\alpha_{1C}\) decreased to 72% and 79% of controls respectively (\(P<0.05\)).
Inhibition of PTK affects the release of a subset of secretory vesicles

Exposure of INS-1 cells to a train of brief depolarizing pulses resulted in a steady increase in cell capacitance, reflecting insulin vesicle exocytosis. This exocytosis was significantly reduced in cells treated with genistein (Fig. 6A). When these results are displayed as the increase in capacitance for each individual pulse, it becomes apparent that exocytosis caused by the initial pulses is significantly affected by PTK inhibition (Fig. 6B). The total increase in capacitance observed in WT cells after this pulse protocol was 240 ± 33 fF (n = 11) compared with 117 ± 23 fF (n = 13) in genistein-treated cells (Fig. 6C) (P < 0.01). This was not due to any reduction in the size of the cells used, as the whole-cell capacitance of WT cells was 9.7 ± 0.7 pF, similar to 10.3 ± 1.0 pF in genistein-treated cells (Fig. 6D). It may be attributed to a reduced Ca^{2+} current amplitude, as the Ca^{2+} current from the first depolarizing step had a mean amplitude of 100.7 ± 17.2 pA in genistein-treated cells, significantly lower than 153.9 ± 14.5 pA in WT cells (Fig. 6E) (P < 0.05). The effect of PTK inhibition on the K_{ATP} and Ca^{2+} current amplitude we have reported appears not to be due to a general downregulation of all ion channel types, as the amplitude and incidence of voltage-dependent Na^{+} currents in INS-1 cells were not altered by genistein treatment. These Na^{+} currents are transient, with full channel inactivation occurring within several milliseconds, making them straightforward to detect and similar in appearance to those previously described in these cells (Merglen et al. 2004). Not all INS-1 cells displayed this current, with 6/19 recorded WT cells containing the Na^{+} current and 5/13 genistein-treated cells displaying it. The amplitude of this Na^{+} current at 0 mV was not altered between treatment groups, with a mean amplitude of 210.8 ± 22.0 pA in WT cells and 201.0 ± 27.2 pA in genistein-treated cells (Fig. 6F).

Discussion

In the present study, we demonstrate that long-term inhibition of the insulin signaling pathway through the inactivation of PTK causes β-cell dysfunction at multiple levels. These include the decreased mRNA expression of Kir6·2 and α1C, resulting in reduced K_{ATP} and Ca^{2+} current amplitude. High glucose-induced action potential firing, Ca^{2+} influx and Ca^{2+} oscillations are also impaired, as is insulin vesicle exocytosis, many of these at least partially due to the observed decrease in the number of functional K_{ATP} and Ca^{2+} channels in the cell membrane.

From our K^{+} current recordings, it is clear that K_{ATP} current amplitude is significantly reduced after genistein treatment. This was illustrated by the diminished effect of tolbutamide on K^{+} current amplitude, the reduced effect of the K_{ATP} channel opener, diazoxide, on membrane potential, the depolarized resting membrane potential and the decreased mRNA expression of Kir6·2 in these cells. Kir6·2 mRNA levels were quantified, as it is the sole α-subunit of K_{ATP} channels present in β-cells (Ashcroft 2000). As the amplitude of tolbutamide-insensitive current measured at negative potentials was the same in both treatment groups, it seems that K_{ATP} channels are the major β-cell K^{+} channel operating at a negative resting potential to have its expression reduced by PTK inhibition.

K_{ATP} channels are open at, and regulate, the resting membrane potential of β-cells (Ashcroft 2000), explaining why the reduction of K_{ATP} channel expression in genistein-treated cells causes increased resting membrane potential. The time to depolarization was also affected by this K_{ATP} channel downregulation, as seen in both current clamp and Ca^{2+} imaging experiments. Insulin is the main stimulator of glucokinase expression...
(Leibiger et al. 2001), and a reduced level of this enzyme could also explain the prolonged latent period before membrane depolarization. This would reduce glucose utilization and cause a slower increase in the ATP/ADP ratio and, thus, a longer time to $K_{ATP}$ channel closure. However, whether genistein treatment has caused a reduction in glucokinase activity or expression in our experiments is unknown.

**Figure 6** Secretion from single INS-1 cells is reduced by PTK inhibition. (A) Example of trace of WT (black trace) and genistein-treated (grey trace) cells subjected to a series of depolarizing pulses to 0 mV from a holding potential of –70 mV (300-ms pulse duration at 1 Hz, shown above trace) and the membrane capacitance measured after each pulse. (B) Mean capacitance change induced by individual voltage pulses. (C) Total capacitance change after completion of the pulse protocol. (D) Starting whole-cell capacitance before the protocol. (E) Amplitude of the $Ca^{2+}$ currents from the first pulse of each series. (F) Amplitude of voltage-dependent $Na^+$ current at 0 mV. Filled columns indicate WT cells ($n=11$ for B, C, D, E and $n=6$ for F); open columns indicate genistein-treated cells ($n=13$ for B, C, D, E and $n=5$ for F). *$P<0.05$; **$P<0.01$. 
Electrophysiologic analysis of both primary β-cells and INS-1 cells also revealed that Ca$^{2+}$ current amplitude is reduced by long-term treatment with genistein. This was confirmed by our expression analysis showing a significant decrease in the mRNA level of the L-type Ca$^{2+}$ channel subunit, α$_{1C}$. L-type Ca$^{2+}$ channels are reported to be responsible for 50–90% of total Ca$^{2+}$ current in mouse β-cells (Plant 1998; Gilon et al. 1997, Schulla et al. 2003). Analysis of β-cell Ca$^{2+}$ currents from mice with a specific ablation of the α$_{1C}$ subunit in these cells reveals that α$_{1C}$ is responsible for all L-type Ca$^{2+}$ current in mouse β-cells (Schulla et al. 2003). The genistein-induced Ca$^{2+}$ channel downregulation underlies the diminished Ca$^{2+}$ influx upon high glucose stimulation and at least partially explains the reduced depolarization-induced insulin vesicle exocytosis in genistein-treated cells. The loss of Ca$^{2+}$ oscillations in these cells is more likely to be due to the reduction in K$_{ATP}$ rather than Ca$^{2+}$ channel expression, as Ca$^{2+}$ oscillations in β-cells result from the reopening of K$_{ATP}$ channels after depolarization (Larsson et al. 1996, Rolland et al. 2002).

The decreased Ca$^{2+}$ currents may also underlie the lack of action potential firing in response to glucose stimulation in genistein-treated cells. Action potentials in primary mouse β-cells are generated by the opening of Ca$^{2+}$ channels (Mears 2004), as voltage-dependent Na$^+$ channels, which typically initiate action potentials in many electrically excitable cells, are inactivated at physiologic membrane potentials in β-cells of this species (Gopel et al. 1999). Blockade of L-type Ca$^{2+}$ channels in β-cells suppresses glucose-induced bursting activity, [Ca$^{2+}$]$_i$ oscillations and insulin secretion (Ashcroft & Rorsman 1989); hence, the decrease in α$_{1C}$ expression and Ca$^{2+}$ current size in genistein-treated cells appears to explain at least partially the loss of these functions in our experiments.

Exocytosis of insulin, as measured by membrane capacitance changes, was significantly reduced in genistein-treated cells. This contrasts with the acute application of genistein, which causes an increase in glucose-stimulated insulin secretion in mouse β-cells (Jonas et al. 1995). However, the authors of this work concluded that this effect of genistein was not due to any direct effect on PTK. The similarity in whole-cell capacitance in both our treatment groups indicates that the reduction in exocytosis we observed is not due to any experimental bias caused by recording from smaller cells. The decrease in secretion appeared to occur most significantly in a subset of early release granules. This granule pool is characterized as a subset of docked secretory vesicles which are immediately ready for release into the extracellular space upon cell excitation (Kanno et al. 2004). L-type Ca$^{2+}$ channel activity has been shown to be highest in β-cells in areas where clusters of vesicles are docked at the cell membrane (Bokvist et al. 1995). This is because the II–III loop of α$_{1C}$ subunits bind with the exocytotic proteins syntaxin 1A, synaptotagmin 1 and SNAP-25 in primary β-cells (Wiser et al. 1999), indicating that α$_{1C}$ subunits are directly involved in the early release of insulin granules. Indeed, in mice in which α$_{1C}$ has been specifically knocked out in β-cells, the first phase of glucose-induced in vitro insulin secretion and the rapid component of exocytosis, as measured by capacitance changes, are significantly reduced (Schulla et al. 2003). It seems most likely that the reduction in the initial components of capacitance increase caused by PTK inhibition in our experiments are due to the concomitant reduction in α$_{1C}$ mRNA expression and Ca$^{2+}$ current amplitude in these genistein-treated cells.

Insulin is thought to act on β-cells in a feed-forward mechanism, given that insulin and insulin mimetics stimulate [Ca$^{2+}$]$_i$ increases and insulin secretion when acutely applied to β-cells (Aspinwall et al. 2000, Roper et al. 2002). This insulin-stimulated insulin secretion occurs via a pathway involving the insulin receptor, the function of which requires PTK activity, insulin receptor substrate-1 (IRS-1) and phosphatidylinositol 3-kinase (Aspinwall et al. 2000). Glucose-induced, first-phase insulin secretion is eliminated in mice with pancreatic β-cell-specific knockout of the insulin receptor (Kulkarni et al. 1999a) and insulin-like growth factor (IGF) receptor (Kulkarni et al. 2002), and in mice with global deletion of IRS-1 (Kulkarni et al. 1999b), highlighting the importance of insulin receptor tyrosine kinase activity and the insulin receptor signaling pathway in β-cell function. IRS-1 knockout mice also display a reduced level of islet insulin content, indicating that the insulin signaling pathway regulates the level of insulin synthesis in β-cells (Kulkarni et al. 1999b). It is postulated that release of vesicles during the first rapid component of exocytosis underlies first-phase, glucose-induced insulin secretion, and that second-phase release reflects the refilling of this early-release vesicle pool (Eason 2000, Mears 2004). Given that a loss of first-phase insulin secretion is among the earliest manifestations of type 2 diabetes, that β-cell specific loss of either α$_{1C}$ Ca$^{2+}$ channel subunit or PTK activity leads to a loss of first-phase, glucose-induced insulin secretion (Kulkarni et al. 1999a, Schulla et al. 2003), as well as early-phase exocytosis in our experiments, the potential importance of our findings becomes apparent. By inhibiting PTK activity, we have interfered with the insulin signaling pathway, resulting in defects in β-cell function similar to those previously mentioned for the knockout of the insulin receptor, IGF receptor and IRS-1. These results reinforce the insulin feed-forward hypothesis that insulin is able to regulate the level of β-cell insulin synthesis and secretion and the β-cell response to high blood glucose in an autocrine or paracrine manner.
Our present study shows that long-term inhibition of PTK activity in β-cells increases resting membrane potential, abolishes action potential firing and [Ca^{2+}], oscillations in response to high glucose, and reduces early-phase insulin vesicle exocytosis. All of these outcomes can be at least partially explained by the decreased K_{ATP} and L-type Ca^{2+} channel expression caused by genistein treatment. We have illustrated that an in vitro inhibition of PTK activity leads to β-cell dysfunction at multiple levels, including factors which underlie the loss of first-phase insulin secretion. These results provide evidence that altered PTK activity may be an important contributing factor to β-cell dysfunction and type 2 diabetes.

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