Linoleic acid induces Ca^{2+} -induced inactivation of voltage-dependent Ca^{2+} currents in rat pancreatic β -cells

Dan-Dan Feng^{1,2,*}, Yu-Feng Zhao^{2,*}, Zi-Qiang Luo¹, Damien J Keating² and Chen Chen²

¹Department of Physiology, Xiangya Medical School, Central South University, Changsha, 410078 People's Republic of China

²Prince Henry's Institute of Medical Research, PO Box 5152, Clayton, Victoria 3168, Australia

(Correspondence should be addressed to C Chen; Email: chen.chen@princehenrys.org)

*(D-D Feng and Y-F Zhao contributed equally to this work)

Abstract

Free fatty acids (FFAs) regulate insulin secretion in a complex pattern and induce pancreatic β -cell dysfunction in type 2 diabetes. Voltage-dependent Ca^{2+} channels (VDCC) in β -cells play a major role in regulating insulin secretion. The aim of present study is to clarify the action of the FFA, linoleic acid, on VDCC in β -cells. The VDCC current in primary cultured rat β -cells were recorded under nystatin-perforated whole-cell recording configuration. The VDCC was identified as high-voltage-gated Ca^{2+} channels due to there being no difference in current amplitude under holding potential between -70 and -40 mV. Linoleic acid ($10\ \mu\text{M}$) significantly inhibited VDCC currents in β -cells, an effect which was fully reversible upon washout. Methyl-linoleic acid, which does not activate G protein coupled receptor (GPR)40, neither did alter VDCC current in rat β -cells nor did influence linoleic acid-induced inhibition of

VDCC currents. Linoleic acid-induced inhibition of VDCC current was not blocked by preincubation of β -cells with either the specific protein kinase A (PKA) inhibitor, H89, or the PKC inhibitor, chelerythrine. However, pretreatment of β -cells with thapsigargin, which depletes intracellular Ca^{2+} stores, completely abolished linoleic acid-induced decrease in VDCC current. Measurement of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) illustrated that linoleic acid induced an increase in $[\text{Ca}^{2+}]_i$ and that thapsigargin pretreatment inhibited this increase. Methyl-linoleic acid neither did induce increase in $[\text{Ca}^{2+}]_i$ nor did it block linoleic acid-induced increase in $[\text{Ca}^{2+}]_i$. These results suggest that linoleic acid stimulates Ca^{2+} release from intracellular Ca^{2+} stores and inhibits VDCC currents in rat pancreatic β -cells via Ca^{2+} -induced inactivation of VDCC.

Journal of Endocrinology (2008) **196**, 377–384

Introduction

Pancreatic β -cells secrete insulin in response to stimulation by nutrients such as glucose and free fatty acids (FFAs). Glucose-stimulated insulin secretion (GSIS) plays an important role in maintaining glucose homeostasis. In contrast, the effects of FFAs on insulin secretion are more complex and less clearly understood. FFAs acutely enhance GSIS, whereas chronic exposure of pancreatic islets to FFAs impairs GSIS (Mason *et al.* 1999, Zraika *et al.* 2002, Haber *et al.* 2003). It has been reported that metabolism of FFAs in β -cells is required for this acute amplification effect on insulin secretion (Malaisse *et al.* 1985, Warnotte *et al.* 1994). Recently, the G-protein-coupled membrane receptor, GPR40, has been identified in β -cells, and further reports showed that FFAs enhance GSIS in a GPR40-dependent manner (Briscoe *et al.* 2003, Itoh *et al.* 2003, Schnell *et al.* 2007).

Pancreatic β -cells are excitable cells expressing many types of ion channels. Activation of voltage-dependent Ca^{2+} channels (VDCC) plays an important role in GSIS; however, the role of VDCC in FFA-induced insulin secretion is not well studied or understood. Some reports suggest that activation of VDCC may be responsible for the FFA-induced increase in $[\text{Ca}^{2+}]_i$ in β -cells

(Remizov *et al.* 2003, Fujiwara *et al.* 2005). Increases in $[\text{Ca}^{2+}]_i$ have an inhibitory effect on VDCC currents in many cell types including pancreatic β -cells, commonly known as Ca^{2+} -induced VDCC inactivation (Plant 1988, Findlay 2004, Yang & Berggren 2005). To further study the action of FFAs on insulin secretion, we investigated the effects of linoleic acid on VDCC in β -cells. The results illustrate an inhibitory effect of linoleic acid on VDCC currents resulting from an increase in $[\text{Ca}^{2+}]_i$ caused by linoleic acid-induced release of Ca^{2+} from intracellular stores. Thus, linoleic acid appears to cause the release of stored intracellular Ca^{2+} resulting in Ca^{2+} -induced VDCC inactivation.

Materials and Methods

Chemicals

Linoleic acid and methyl-linoleic acid were purchased from Sigma. Histopaque-1077, dispase, collagenase (type V), RPMI-1640, nifedipine, nystatin, and all reagents for bath and pipette solutions were purchased from Sigma. Chelerythrine and H89 were purchased from Calbiochem (Alexandria, NSW, Australia).

TTX was from Alomone Laboratories (Jerusalem, Israel). Fetal bovine serum, HEPES and penicillin/streptomycin were from Thermo Electron (Melbourne, VIC, Australia).

Preparation and culture of rat pancreatic β -cells

Pancreatic islets were isolated from 10 to 12 week-old male Sprague–Dawley rats as previously described (Zhao *et al.* 2005). Briefly, rats were obtained from Monash University and killed by CO₂ inhalation as approved by Animal Ethics Committee of Monash University. The pancreas was inflated by injecting 10 ml collagenase solution (0.5 mg/ml in Hank's solution) into it through the bile duct. The pancreas was isolated and digested at 37 °C for 30 min, and then dispersed. The islets were separated by Histopaque-1077 density gradient centrifugation and collected for cell isolation. The islets were dispersed into single cells by digestion with dispase solution (1 mg/ml in Ca²⁺-free Hank's solution). The islet cells were plated onto 35 mm plastic dishes for electrophysiological recordings and onto glass coverslips coated with 0.01% poly-L-lysine for [Ca²⁺]_i measurement experiments. The islet cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 g/ml streptomycin in a humidified atmosphere of 95% air and 5% CO₂. The culture medium was changed every 2 days. The experiments were performed during day 3–6 in culture.

Electrophysiological recording

The cells were washed with bath solution before the recording. Recordings were made using perforated whole-cell patch clamp configuration. Electrodes were pulled by a Sutter P-87 microelectrode puller from borosilicate micropipettes and had an initial input resistance of 3–5 M Ω . All recordings were performed using a Axopatch 200A amplifier (Axon Instrument). The bath solution for Ca²⁺ currents recording was composed of (mM): 120 NaCl, 20 TEA-Cl, 4.7 KCl, 2.6 CaCl₂, 1.2 MgCl₂, 1.2 Na₂HPO₄, 1 NaHCO₃, 10 glucose, and 5 HEPES (pH 7.4 with NaOH). Tetrodotoxin (TTX) (10⁻⁷ M) was added into the bath solution before recording. The pipette solution for Ca²⁺ current recording was composed of (mM): 76 Cs₂SO₄, 30 CsCl, 10 NaCl, 8 MgSO₄, and 20 HEPES (pH 7.3 with NaOH). Membrane perforation was achieved by the presence of 200 μ g/ml nystatin in the pipette solution. After formation of a high-resistance seal, the pipette was held at a potential of -70 mV. Access to the interior of the cell was considered adequate once the series resistance was lower than 30 M Ω . The pipette capacitance and whole-cell capacitance were compensated before experiments. The β -cells were identified by cell size and cell membrane capacitance. Immunocytochemical staining of islets cells showed that β -cells are much bigger in diameter than other types of cells, and electrophysiological experiments revealed that β -cells have a whole-cell capacitance larger than other types of cells. The mean sizes of β - and α -cells are 5.5 $\pm$ 0.3 and 2.8 $\pm$ 0.1 pF respectively (Gopel *et al.* 2000, Leung *et al.* 2005). The criterion

for β -cell identification in our experiment is that the whole-cell capacitance of the clamped cells is over 6 pF.

Measurement of [Ca²⁺]_i

Islet cells were loaded with 1 μ M Fura-2/AM in RPMI-1640 medium for 30 min at 37 °C. Cells were subsequently rinsed with bath solution and kept for 20 min in this solution to allow full de-esterification of the dye and to equilibrate the cells. Fluorescence measurements were performed using a dual excitation microfluorescence spectro-analysis system and data were recorded using Axoscope 8.2 (Axon Instrument, Sunnyvale, CA, USA). Fura-2 was excited at 340 and 380 nm, with fluorescence emission detected at 510 nm. [Ca²⁺]_i was calculated according to the formula described by Grynkiewicz *et al.* (1985). [Ca²⁺]_i (nM), $K_d \times (F_o/F_s) \times (R - R_{min}) / (R_{max} - R)$, where K_d , the Fura-2 dissociation constant (225 nM), F_o , the 380 nm fluorescence in the absence of calcium, F_s , the 380 nm fluorescence with saturating calcium, R , the 340/380 nm fluorescence ratio, R_{max} , the 340/380 nm ratio with saturating calcium, and R_{min} , the 340/380 nm ratio in the absence of calcium. F_o/F_s , R_{max} , and R_{min} were determined in the recorded cells. Briefly, the cells were permeabilized by 20 μ M ionomycin for 10 min to allow sufficient extracellular Ca²⁺ entry, and the resulting 340/380 nm ratio is R_{max} . After a steady value of R_{max} had been obtained, the R_{min} value was determined by chelating Ca²⁺ with 8 mM EGTA. All records were corrected for autofluorescence of unloaded cells at each wavelength before the ratio was calculated. Cells were constantly perfused at a rate of 3 ml/min. Experimental reagents were dissolved in the bath solution just before the recordings and delivered through a gravity perfusion system. The β -cells were identified by increase in [Ca²⁺]_i in responding to 16 mM glucose stimulation. The experiments of [Ca²⁺]_i measurements were done after 15 min recovery from 16 mM glucose stimulation. The bath solution used for [Ca²⁺]_i measurements was composed of (mM): 140 NaCl, 4.7 KCl, 2.6 CaCl₂, 1.2 MgSO₄, 1 NaHCO₃, 1.2 Na₂HPO₄, 3 glucose and 5 HEPES (pH 7.4 with NaOH).

Statistical analysis

The data are represented as mean $\pm$ S.E.M. for each group. One-way ANOVA was used to analyze the statistical significance between treatment and control groups for calcium current recording and the [Ca²⁺]_i measurements. $P < 0.05$ was taken as the minimum level of significance.

Results

Linoleic acid inhibits VDCC currents in β -cells

The whole-cell VDCC currents were measured by stepping from a holding potential of -70 mV to depolarizing pulses

from -50 to 50 mV in 10 mV increments. These cells were then held at -40 mV and depolarized from -30 to 50 mV in 10 mV increments. VDCC currents were activated at depolarizing potential of -30 mV and reached the maximal level at depolarizing potential of 10 mV. The maximal amplitude and shape of these Ca^{2+} currents was not significantly different when cells were stepped from a holding potential of either -70 or -40 mV, indicating that no low-voltage-gated Ca^{2+} currents but high-voltage-gated Ca^{2+} currents exist in rat β -cells. The cells were held at -70 mV for the following experiments. Application of linoleic acid at a concentration of 10 μM for 2 min induced a significant decrease in the amplitude of VDCC currents. While the amplitude of VDCC currents was reduced, the time course did not change (Fig. 1Aa and b). VDCC currents recovered to control levels after 5 min washout of linoleic acid (Fig. 1Ac). The peak VDCC current amplitude was measured and analyzed at the depolarizing potential of 10 mV. The amplitude of VDCC currents in rat β -cells under control conditions was 106.17 ± 10.47 pA, and it decreased to 50.13 ± 8.696 pA in the presence of 10 μM linoleic acid ($P < 0.01$, $n = 5$). After washout, the VDCC currents recovered to 95.32 ± 7.595 pA (Fig. 1B). Linoleic acid inhibited VDCC currents under both 10 and 5 mM glucose in the same manner. There was not significant difference in the inhibition of VDCC currents by linoleic acid between 10 and 5 mM glucose conditions.

Methyl-linoleic acid does not inhibit VDCC currents or block the effect of linoleic acid

Methyl-linoleic acid is an analog of linoleic acid without binding affinity to the FFA receptor, GPR40. Application of 10 μM methyl-linoleic acid to β -cells for 10 min did not inhibit VDCC currents (Fig. 2Ab). Subsequent application of 10 μM linoleic acid to the same cells for 2 min significantly inhibited VDCC currents (Fig. 2Ac). The maximal amplitude of VDCC currents in each group was 94.84 ± 12.25 pA in control, 91.91 ± 11.73 pA after methyl-linoleic acid, and 46.28 ± 6.25 pA after linoleic acid in the continued presence of methyl-linoleic acid ($P < 0.01$, linoleic acid versus control, $n = 5$, Fig. 2B).

Figure 1 Effect of linoleic acid on VDCC currents. (A) Representative VDCC current traces recorded in rat β -cells (a) in control, (b) after linoleic acid and (c) in recovery after washout. (B) Statistical data of the Ca^{2+} currents measured at the maximal levels of pulses and expressed as mean $\pm$ S.E.M. (** $P < 0.01$ versus control, $n = 5$).

Inhibition of PKC and PKA does not influence the linoleic acid-induced inhibition of VDCC currents

Inhibitors for PKA and protein kinase C (PKC) were used to identify the intracellular signaling pathway activated by linoleic acid to inhibit VDCC currents in rat β -cells. Incubation for 15 min with 10 μM chelerythrine, a broad inhibitor of PKC, did not alter VDCC current amplitude. Additionally, in the continued presence of chelerythrine, linoleic acid inhibited VDCC currents to a similar level to that seen in the absence of chelerythrine. The maximal amplitude of VDCC currents in each group was 133.19 ± 4.269 pA in control, 137.98 ± 5.703 pA after chelerythrine, and 61.84 ± 3.689 pA after linoleic acid in the presence of chelerythrine (Fig. 3A, $P < 0.01$ linoleic acid versus control, $n = 5$). H89 at 1 μM , an inhibitor of PKA, also did not change the degree of VDCC current inhibition induced by linoleic acid in β -cells. The maximal VDCC current amplitude in each group was 132.34 ± 12.248 pA in control, 136.86 ± 9.731 pA after H89, and 48.41 ± 11.732 pA after linoleic acid in combination with H89 (Fig. 3B, $P < 0.01$ linoleic acid versus control, $n = 5$).

Thapsigargin treatment blocks linoleic acid-induced inhibition of VDCC currents

It has been previously reported that FFAs stimulate Ca^{2+} release from intracellular Ca^{2+} stores (Itoh *et al.* 2003, Fujiwara *et al.* 2005). We therefore tested the effects of intracellular Ca^{2+} release on the VDCC currents activation. β -Cells were treated with 1 μM thapsigargin for 30 min prior to patch clamping, cells were patched and VDCC currents recorded. After thapsigargin treatment, 10 μM linoleic acid up to 5 min could not inhibit VDCC currents further. The maximal amplitude of VDCC currents was 89.29 ± 14.33 pA in control and 89.89 ± 18.69 pA after linoleic acid (Fig. 4, $n = 5$).

Thapsigargin treatment eliminates the linoleic acid-induced increase in $[\text{Ca}^{2+}]_i$ in β -cells

To confirm the block of increase in $[\text{Ca}^{2+}]_i$ after thapsigargin treatment, we measured $[\text{Ca}^{2+}]_i$ in β -cells. Linoleic acid

Figure 2 Effect of methyl-linoleic acid on VDCC currents. Methyl-linoleic acid did not influence VDCC currents or linoleic acid-induced inhibition of VDCC currents. (A) Representative VDCC current traces recorded in rat β -cells (a) in control, (b) after methyl-linoleic acid and (c) after linoleic acid with methyl-linoleic acid. (B) Statistical data of the VDCC currents measured the maximal levels of pulses and expressed as mean $\pm$ S.E.M. (** $P < 0.01$ versus control, $n = 5$).

stimulated a quick increase in $[\text{Ca}^{2+}]_i$ in β -cells (Fig. 5A, $n = 10$). Thapsigargin pretreatment for 30 min blocked linoleic acid-stimulated increase in $[\text{Ca}^{2+}]_i$ in β -cells (Fig. 5B, $n = 6$). Changes in $[\text{Ca}^{2+}]_i$ induced by linoleic acid were calculated as the mean value of $[\text{Ca}^{2+}]_i$ in 5 min after linoleic acid stimulation. There was no significant difference in the basal level of $[\text{Ca}^{2+}]_i$ between control cells and those treated with thapsigargin for 30 min (Fig. 5C). The changes in $[\text{Ca}^{2+}]_i$ after linoleic acid stimulation were eliminated by thapsigargin treatment (73.06 ± 5.007 nM in control group, $n = 10$, and 25.88 ± 2.441 nM in thapsigargin group, $n = 6$, $P < 0.01$, Fig. 5D).

Methyl-linoleic acid does not induce an increase in $[\text{Ca}^{2+}]_i$ and does not inhibit the linoleic acid-induced increase in β -cell $[\text{Ca}^{2+}]_i$

Methyl-linoleic acid, at concentrations ranging from 10 to 50 μM did not stimulate any increase in $[\text{Ca}^{2+}]_i$ in β -cells

(Fig. 6A, $n = 6$). There was no significant difference in $[\text{Ca}^{2+}]_i$ levels after methyl-linoleic acid stimulation (Fig. 6C). Additionally, methyl-linoleic acid did not affect the linoleic acid-induced increase in $[\text{Ca}^{2+}]_i$ in β -cells (Fig. 6B, $n = 4$). Under the presence of methyl-linoleic acid, linoleic acid stimulated significant increase in $[\text{Ca}^{2+}]_i$ in β -cells (Fig. 6D). There was no significant difference to changes in $[\text{Ca}^{2+}]_i$ between control cells and those treated with methyl-linoleic acid (73.06 ± 5.007 nM in control group, $n = 10$, and 75.07 ± 5.871 nM in methyl-linoleic acid group, $n = 4$).

U73122 treatment blocked linoleic acid-induced inhibition of VDCC current and increase in $[\text{Ca}^{2+}]_i$ in β -cells

It has been suggested that FFAs activate GPR40 and GPR40 activates phospholipase C (PLC) to induce calcium release by producing IP3 (Briscoe *et al.* 2003, Fujiwara *et al.* 2005). We tested the effect of blockade of PLC by U73122 on linoleic

Figure 3 Effect of protein kinase blockers on linoleic acid-induced inhibition of VDCC currents. (A) 10 μM chelerythrine, a PKC inhibitor, did not modify VDCC currents and linoleic acid-induced inhibition of VDCC currents (** $P < 0.01$ versus control, $n = 5$). (B) 1 μM H89, a PKA inhibitor, did not modify VDCC currents and linoleic acid-induced inhibition of VDCC currents (** $P < 0.01$ versus control, $n = 5$). Data were presented as mean $\pm$ S.E.M.

Figure 4 Effect of thapsigargin treatment on the linoleic acid-induced reduction in VDCC currents. After the cells were treated with 1 μM thapsigargin for 30 min, linoleic acid (10 μM) did not inhibit the VDCC currents in β -cells ($n=5$). Data were presented as mean $\pm$ S.E.M.

acid-induced inhibition of VDCC and the increase in $[\text{Ca}^{2+}]_i$ in β -cells. The VDCC currents were inhibited to $47.26\% \pm 6.899\%$ of basal levels by linoleic acid. After U73122 treatment (5 μM for 20 min), the inhibitory effect of linoleic acid on VDCC currents was totally blocked. (Fig. 7A, $P < 0.01$ versus control, $n=5$ each group). Linoleic acid stimulated significant increase in $[\text{Ca}^{2+}]_i$ levels, and this increase was totally blocked by 5 μM U73122 treatment for 20 min. The $[\text{Ca}^{2+}]_i$ change 8B was $258.87\% \pm 24.89\%$ in the control, which decreased to $110.77\% \pm 8.89\%$ in U73122 group (Fig. 7B, $P < 0.01$ versus control, $n=8$).

Discussion

The present study demonstrates that linoleic acid inhibits VDCC currents in β -cells via Ca^{2+} -induced inactivation of VDCC. The elimination of the linoleic acid-stimulated increase in $[\text{Ca}^{2+}]_i$ in β -cells by the depletion of intracellular Ca^{2+} stores indicates that linoleic acid stimulates an increase in $[\text{Ca}^{2+}]_i$ by causing Ca^{2+} release from intracellular Ca^{2+} stores. The inhibition of linoleic acid-stimulated increase in $[\text{Ca}^{2+}]_i$ also eliminated the inhibitory effects of linoleic acid on Ca^{2+} currents in β -cells, suggesting that linoleic acid inhibits VDCC currents indirectly by inducing Ca^{2+} release and subsequently causing Ca^{2+} -dependent inactivation of VDCC.

$[\text{Ca}^{2+}]_i$ is known to have feedback regulatory effects on VDCC. The Ca^{2+} -dependent inactivation of VDCC is a common characteristic of VDCC and is considered an important mechanism protecting against Ca^{2+} overload (Budde *et al.* 2002, Findlay 2004). Calcium-dependent inactivation of VDCC has been described in mouse and human pancreatic β -cells (Plant 1988, Kelly *et al.* 1991). The regulatory domain of the $\text{Ca}_v1.2$ subunit is suggested to bind Ca^{2+} and calmodulin, and the resulting conformational changes upon this binding result in Ca^{2+} -dependent VDCC inactivation (Soldatov 2003). Our present study indicates that an increase in $[\text{Ca}^{2+}]_i$ in β -cells resulting from intracellular Ca^{2+} release also leads to inactivation of Ca^{2+} channels. Both activation of GPR40 and FFA metabolites can activate PKC (Poitout 2003) and GPR40 may also activate PKA

Figure 5 Linoleic acid-induced increase in $[\text{Ca}^{2+}]_i$ and the effects of thapsigargin on linoleic acid-induced increase in $[\text{Ca}^{2+}]_i$ in β -cells. Linoleic acid induced an increase in $[\text{Ca}^{2+}]_i$ in β -cells (A, $n=10$). Thapsigargin pretreatment inhibited linoleic acid-induced increase in $[\text{Ca}^{2+}]_i$ in β -cells (B, $n=6$). The statistical results showed that the basal levels of $[\text{Ca}^{2+}]_i$ in control and treatment groups were not significantly different (C). Changes in $[\text{Ca}^{2+}]_i$ induced by linoleic acid were calculated as the mean value of $[\text{Ca}^{2+}]_i$ in 5 min after linoleic acid stimulation. The changes in $[\text{Ca}^{2+}]_i$ after linoleic acid stimulation were eliminated by thapsigargin treatment (D). Data were presented as mean $\pm$ S.E.M.

Figure 6 Effects of methyl-linoleic acid on $[Ca^{2+}]_i$ in β -cells. Methyl-linoleic acid did not induce an increase in $[Ca^{2+}]_i$ in β -cells. (A) The statistic results showed that the mean values of $[Ca^{2+}]_i$ in 5 min after methyl-linoleic acid stimulation were not different from the control (C, $n=6$). Methyl-linoleic acid did not inhibit linoleic acid-induced increase in $[Ca^{2+}]_i$ in β -cells (B). The statistic results showed that the mean values of $[Ca^{2+}]_i$ in 5 min after linoleic acid stimulation on the top of methyl-linoleic acid were significantly increased (D, $n=4$). Data were presented as mean $\pm$ S.E.M.

(Feng *et al.* 2006). H89 is a cell-permeable and potent inhibitor of PKA with IC₅₀ of 50 nM (Chijiwa *et al.* 1990). Chelerythrine is a potent and selective PKC inhibitor with IC₅₀ of 0.66 μM (Herbert *et al.* 1990). By inhibiting these PKs by H89 and chelerythrine, we found the linoleic acid-induced inhibition of Ca²⁺ currents is not due to the inhibition and the activation of either PKA or PKC. We reported that linoleic acid reduced voltage-gated potassium channels via PKA signaling pathway (Feng *et al.* 2006). The present study showed that the mechanism of inhibition of VDCC currents by linoleic acid is different from that of inhibition of voltage-gated potassium channels. Moreover, since linoleic acid inhibited VDCC, the significance of reduction of voltage-gated potassium channels in insulin secretion should be reevaluated. From the present study, it seems that intracellular calcium release stimulated by linoleic acid may be responsible for linoleic acid-stimulated insulin secretion.

Inhibition of Ca²⁺ channels by linoleic acid is a novel mechanism of the effects of FFAs on insulin secretion and GSIS. Glucose stimulates insulin secretion by both K_{ATP} channel-dependent and K_{ATP} channel-independent pathways (Henquin 2000). In the K_{ATP} channel-dependent pathway, glucose is metabolized in β-cells to generate ATP. This increase in the ATP/ADP ratio leads to the closure of K_{ATP} channels and cell depolarization which activates Ca²⁺ channels and results in Ca²⁺ influx. This increase in $[Ca^{2+}]_i$ triggers the exocytosis of insulin-containing vesicles. Ca²⁺ channels are necessary for GSIS (Rorsman 1997). Inhibition of Ca²⁺ channels would therefore attenuate GSIS

occurring via the K_{ATP} channel-dependent pathway. This may be one reason for the inhibition of GSIS by FFAs.

Our results indicate that FFAs stimulate an increase in $[Ca^{2+}]_i$ by releasing Ca²⁺ from thapsigargin-sensitive stores. Some previous reports showed that the L-type VDCC blocker nifedipine can inhibit FFA-induced increase in $[Ca^{2+}]_i$ and suggest that the activation of VDCC may be involved in the FFA-induced increase in $[Ca^{2+}]_i$ in β-cells (Remizov *et al.* 2003). However, our results showed that linoleic acid inhibited Ca²⁺ currents in a Ca²⁺-dependent manner. Thapsigargin used by Fujiwara *et al.* (2005) was at the concentration of 0.2 μM at 12 mM glucose for about 10 min. In contrast, we treated the cells with thapsigargin at 1 μM at 3 mM glucose for more than 30 min to fully deplete intracellular calcium stores. This difference in experimental condition may be one reason for the discrepancy. Considering the dependence on intracellular calcium and extracellular calcium for FFAs-induced increase in $[Ca^{2+}]_i$ in β-cells, we provide the possible involvement of calcium-induced calcium release mechanism for FFAs-induced increase in $[Ca^{2+}]_i$ in β-cells.

The effects of linoleic acid on VDCC may be mediated indirectly by GPR40. Olofsson *et al.* (2004) reported that palmitate increases VDCC at low concentration, but decreases it at high concentration in mouse β-cells. Because the palmitate they used was prepared by addition of 1% BSA, and BSA at the level above 0.1% blocked the action of FFAs on plasma membrane receptor GPR40 (Itoh *et al.* 2003), the effects observed by Olofsson would not be GPR40-mediated effects. In our study, the linoleic acid used is without BSA and can

Figure 7 Effects of U73122 treatment on linoleic acid-induced inhibition of VDCC currents and increase in $[\text{Ca}^{2+}]_i$ in β -cells. (A) U73122 treatment totally blocked linoleic acid-induced inhibition of VDCC currents (** $P < 0.01$ versus linoleic acid, $n = 5$). (B) U73122 treatment totally blocked linoleic acid-induced increase in $[\text{Ca}^{2+}]_i$ in β -cells (** $P < 0.01$ versus linoleic acid, $n = 8$). Data were presented as mean $\pm$ S.E.M.

activate GPR40. In the present study, linoleic acid demonstrated a quick effect on Ca^{2+} currents, suggesting the direct action on plasma membrane receptor GPR40. In addition, depletion of intracellular Ca^{2+} stores by thapsigargin eliminated the quick increase in $[\text{Ca}^{2+}]_i$ in β -cells and blocked the effects of linoleic acid on inhibition of Ca^{2+} channels. This can be easily explained by the fact that linoleic acid-activated GPR40 is linked to PLC and stimulated Ca^{2+} release from thapsigargin-sensitive Ca^{2+} stores. Moreover, methyl-linoleic acid, which has a similar structure to linoleic acid but without affinity to GPR40 (Itoh *et al.* 2003), did not stimulate an increase in $[\text{Ca}^{2+}]_i$ or inhibit Ca^{2+} currents in β -cells. It also did not inhibit the linoleic acid-induced increase in $[\text{Ca}^{2+}]_i$ in β -cells or block linoleic acid-induced inhibition of VDCC currents. Finally, we observed that PLC inhibitor U73122 totally blocked linoleic acid-induced inhibition of VDCC currents and increase in $[\text{Ca}^{2+}]_i$ in β -cells. It has been suggested that GPR40 is linked to PLC (Briscoe *et al.* 2003, Fujiwara *et al.* 2005). The present study

further confirmed it and supports that the effects of linoleic acid on VDCC currents and $[\text{Ca}^{2+}]_i$ are mediated by GPR40 that linked to PLC. Latour *et al.* (2007) generated GPR40 knockout mice and observed about 50% reduction of lipid-stimulated insulin secretion. Considering the inhibitory effects of GPR40 activation on inhibition of VDCC we observed, there may be an offset of the inhibitory effects of GPR40 knockout on insulin secretion. GPR40 may be responsible for more than 50% of lipid-stimulated insulin secretion.

This study gives a new insight into the mechanism of FFA-regulated insulin secretion. It has been shown that FFAs stimulate insulin secretion and inhibit GSIS, and the intracellular metabolites of FFAs are proposed to mediate the inhibitory effects of FFAs on GSIS (Warnotte *et al.* 1994, Haber *et al.* 2003). Our present study firstly indicates that GPR40 activation by FFAs may be involved in the inhibitory effects of FFAs on GSIS by inducing inhibition of VDCC currents. On the other hand, the present study supports that FFA-induced insulin secretion results from an increase in $[\text{Ca}^{2+}]_i$ originating from intracellular Ca^{2+} release. The basal increase in insulin levels and inhibition of GSIS are characters of β -cell dysfunction in type 2 diabetes and can be explained by the stimulation of calcium release and inhibition of VDCC by FFAs.

Acknowledgements

This work was supported by Australian NHMRC and Eli Lilly Endocrinology Program (to C C). We are grateful to Ms D Arnold for editorial help. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

- Briscoe CP, Tadayyon M, Andrews JL, Benson WG, Chambers JK, Eilert MM, Ellis C, Elshourbagy NA, Goetz AS, Minnick DT *et al.* 2003 The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids. *Journal of Biological Chemistry* **278** 11303–11311.
- Budde T, Meuth S & Pape HC 2002 Calcium-dependent inactivation of neuronal calcium channels. *Nature Reviews, Neuroscience* **3** 873–883.
- Chijiwa T, Mishima A, Hagiwara M, Sano M, Hayashi K, Inoue T, Naito K, Toshioka T & Hidaka H 1990 Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-bromocinnamyl)-amino]ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. *Journal of Biological Chemistry* **265** 5267–5272.
- Feng DD, Luo Z, Roh SG, Hernandez M, Tawadros N, Keating DJ & Chen C 2006 Reduction in voltage-gated K^+ currents in primary cultured rat pancreatic beta-cells by linoleic acids. *Endocrinology* **147** 674–682.
- Findlay I 2004 Physiological modulation of inactivation in L-type Ca^{2+} channels: one switch. *Journal of Physiology* **554** 275–283.
- Fujiwara K, Maekawa F & Yada T 2005 Oleic acid interacts with GPR40 to induce Ca^{2+} signaling in rat islet beta-cells: mediation by PLC and L-type Ca^{2+} channel and link to insulin release. *American Journal of Physiology, Endocrinology and Metabolism* **289** E670–E677.
- Gopel SO, Kanno T, Barg S, Weng XG, Gromada J & Rorsman P 2000 Regulation of glucagon release in mouse β -cells by KATP channels and inactivation of TTX-sensitive Na^+ channels. *Journal of Physiology* **528** 509–520.

- Gryniewicz G, Poenie M & Tsien RY 1985 A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry* **260** 3440–3450.
- Haber EP, Ximenes HM, Procopio J, Carvalho CR, Curi R & Carpinelli AR 2003 Pleiotropic effects of fatty acids on pancreatic beta-cells. *Journal of Cellular Physiology* **194** 1–12.
- Henquin JC 2000 Triggering and amplifying pathways of regulation of insulin secretion by glucose. *Diabetes* **49** 1751–1760.
- Herbert JM, Augereau JM, Gleye J & Maffrand JP 1990 Chelerythrine is a potent and specific inhibitor of protein kinase C. *Biochemical and Biophysical Research Communications* **172** 993–999.
- Itoh Y, Kawamata Y, Harada M, Kobayashi M, Fujii R, Fukusumi S, Ogi K, Hosoya M, Tanaka Y, Uejima H *et al.* 2003 Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40. *Nature* **422** 173–176.
- Kelly RP, Sutton R & Ashcroft FM 1991 Voltage-activated calcium and potassium currents in human pancreatic beta-cells. *Journal of Physiology* **443** 175–192.
- Latour MG, Alquier T, Oseid E, Tremblay C, Jetton TL, Luo J, Lin DC & Poitout V 2007 GPR40 is necessary but not sufficient for fatty acid stimulation of insulin secretion *in vivo*. *Diabetes* **56** 1087–1094.
- Leung YM, Ahmed I, Sheu L, Tsushima RG, Diamant NE, Hara M & Gaisano HY 2005 Electrophysiological characterization of pancreatic islet cells in the mouse insulin promoter–green fluorescent protein mouse. *Endocrinology* **146** 4766–4775.
- Malaisse WJ, Malaisse-Lagae F, Sener A & Hellerstrom C 1985 Participation of endogenous fatty acids in the secretory activity of the pancreatic β -cell. *Biochemical Journal* **227** 995–1002.
- Mason TM, Goh T, Tchipashvili V, Sandhu H, Gupta N, Lewis GF & Giacca A 1999 Prolonged elevation of plasma free fatty acids desensitizes the insulin secretory response to glucose *in vivo* in rats. *Diabetes* **48** 524–530.
- Olofsson CS, Salehi A, Holm C & Rorsman P 2004 Palmitate increases L-type Ca²⁺ currents and the size of the readily releasable granule pool in mouse pancreatic beta-cells. *Journal of Physiology* **557** 935–948.
- Plant TD 1988 Properties and calcium-dependent inactivation of calcium currents in cultured mouse pancreatic β -cells. *Journal of Physiology* **404** 731–747.
- Poitout V 2003 The ins and outs of fatty acids on the pancreatic beta cell. *Trends in Endocrinology and Metabolism* **14** 201–203.
- Remizov O, Jakubov R, Dufer M, Krippeit Drews P, Drews G, Waring M, Brabant G, Wienbergen A, Rustenbeck I & Schofl C 2003 Palmitate-induced Ca²⁺-signaling in pancreatic beta-cells. *Molecular and Cellular Endocrinology* **212** 1–9.
- Rorsman P 1997 The pancreatic beta-cell as a fuel sensor: an electrophysiologist's viewpoint. *Diabetologia* **40** 487–495.
- Schnell S, Schaefer M & Schofl C 2007 Free fatty acids increase cytosolic free calcium and stimulate insulin secretion from beta-cells through activation of GPR40. *Molecular and Cellular Endocrinology* **263** 173–180.
- Soldatov NM 2003 Ca²⁺ channel moving tail: link between Ca²⁺-induced inactivation and Ca²⁺ signal transduction. *Trends in Pharmacological Sciences* **24** 167–171.
- Warnotte C, Gilon P, Nenquin M & Henquin JC 1994 Mechanisms of the stimulation of insulin release by saturated fatty acids. A study of palmitate effects in mouse beta-cells. *Diabetes* **43** 703–711.
- Yang SN & Berggren PO 2005 Beta-cell CaV channel regulation in physiology and pathophysiology. *American Journal of Physiology, Endocrinology and Metabolism* **288** E16–E28.
- Zhao YF, Keating DJ, Hernandez M, Feng DD, Zhu Y & Chen C 2005 Long-term inhibition of protein tyrosine kinase impairs electrophysiologic activity and a rapid component of exocytosis in pancreatic beta-cells. *Journal of Molecular Endocrinology* **35** 49–59.
- Zraika S, Dunlop M, Proietto J & Andrikopoulos S 2002 Effects of free fatty acids on insulin secretion in obesity. *Obesity Reviews* **3** 103–112.

Received in final form 19 November 2007

Accepted 23 November 2007

Made available online as an Accepted Preprint
23 November 2007