GW9508 inhibits insulin secretion by activating ATP-sensitive potassium channels in rat pancreatic β -cells

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Abstract

GW9508 is an agonist of G protein-coupled receptor 40 (GPR40) that is expressed in pancreatic β -cells and is reported to regulate insulin secretion. However, the effects of GW9508 on pancreatic β -cells in primary culture have not been well investigated. This study measured the acute effects of GW9508 on insulin secretion from rat pancreatic islets in primary culture, and the insulin secretion-related events such as the changes in membrane potential, ATP-sensitive potassium currents (K_{ATP} currents), and intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) of rat islet β -cells were also recorded. GW9508 (10–40 μ M) did not influence basal insulin levels at 2 mM glucose, but it (above 20 μ M) significantly inhibited 5 and 15 mM glucose-stimulated insulin secretion (GSIS). GW9508 did not inhibit insulin secretion stimulated by tolbutamide, the closer of K_{ATP} channels. GW9508 activated K_{ATP} channels and blocked the membrane depolarization and the increase in $[Ca^{2+}]_i$ that were stimulated by glucose. GW9508 itself stimulated a transient increase in $[Ca^{2+}]_i$, which was fully blocked by depletion of intracellular Ca^{2+} stores with thapsigargin or by inhibition of phospholipase C (PLC) activity with U73122. GW9508-induced activation of K_{ATP} channels was only partly inhibited by U73122 treatment. In conclusion, although it stimulates a transient release of Ca^{2+} from intracellular Ca^{2+} stores via activation of PLC, GW9508 inhibits GSIS by activating K_{ATP} channels probably in a distal step to GPR40 activation in rat β -cells.

Key Words

- ▶ GW9508
- ▶ insulin
- ▶ K_{ATP} channels
- ▶ β -cells

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Introduction

GW9508 (3-(4-(((3-(phenyloxy)phenyl)methyl)amino)phenyl)propanoic acid) was first identified as an agonist for free fatty acid receptors (FFARs) such as G protein-coupled receptor 40 (GPR40) and GPR120 from a high-throughput screen of the GlaxoSmithKline chemical collection in 2006 (Briscoe *et al.* 2006). In previous reports about FFARs actions on pancreatic

β -cells, FFAs were commonly used as agonists of FFARs, but the effects of GW9508 on β -cell function were seldom reported. There are only reports showing that GW9508 potentiated glucose-stimulated insulin secretion (GSIS) in insulinoma cell lines such as MIN6 cells and INS-1E cells (Briscoe *et al.* 2006, Yang *et al.* 2010). And it was shown that GW9508 does not influence insulin secretion in rat

and mouse islets in primary culture (Briscoe *et al.* 2006). The detailed effects of GW9508 on the function of β -cells in primary culture are not well known.

In our previous study, we found that GW9508 activates ATP-sensitive potassium channels (K_{ATP} channels) on rat β -cells in primary culture (Zhao *et al.* 2008). The K_{ATP} channel is a key controller to β -cell excitability and GSIS. It is known that pancreatic β -cells metabolize glucose to generate ATP from ADP, and the increased ATP:ADP ratio closes K_{ATP} channels and leads to membrane depolarization and a concomitant rise in the levels of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and insulin secretion (Ashcroft *et al.* 1994, Rorsman 1997, Drews *et al.* 2010, Rorsman *et al.* 2011). In the present study, we further investigated the effects of GW9508 on insulin secretion, K_{ATP} channels, and $[Ca^{2+}]_i$ levels in primary rat islets and β -cells. It was found that GW9508 activates K_{ATP} channels and inhibits GSIS.

Materials and methods

Materials

GW9508 was obtained from GlaxoSmithKline and Sigma. Histopaque-1077, Dispase, collagenase (type V), DNase I, BSA, RPMI-1640, thapsigargin, tolbutamide, and all reagents for bath solution and pipette solutions were purchased from Sigma. U73122 was obtained from Calbiochem (San Diego, CA, USA). Fura-2/AM was purchased from Invitrogen. FCS, HEPES, and penicillin/streptomycin were obtained from Gibco. QIAamp DNA Investigator kits were obtained from Qiagen and the Qubit dsDNA HS assay kits were purchased from Invitrogen.

Preparation and culture of rat pancreatic β -cells

Sprague Dawley rats (8–10 weeks old) were purchased from the Animal House of The University of Queensland (UQ). The animal experiment was reviewed and approved by the Animal Care and Use Committee of UQ. Pancreatic islets were isolated from 10- to 12-week-old male Sprague Dawley rats as described previously (Zhao *et al.* 2008). Briefly, rats were killed and the pancreas was inflated by injecting 10 ml collagenase solution into it through the bile duct. The collagenase solution was composed of 0.5 mg/ml collagenase, 0.1 mg/ml DNase I, and 1 mg/ml BSA in Hank's Balanced Salt Solution (HBSS). The pancreas were collected and digested at 37 °C for 30 min in stationary state and then they were dispersed by shaking. The islets were separated by Histopaque-1077 density gradient centrifugation and collected under microscope.

Insulin assay was done using the isolated islets. For single-cell recording, the islets were dispersed into single cells by digestion with dispase solution. Dispase solution was composed of 1 mg/ml dispase, 0.1 mg/ml DNase I, and 1 mg/ml BSA in Ca^{2+} -free HBSS. The islet cells were plated into 35 mm dishes or onto glass coverslips coated with 0.01% poly-L-lysine for electrophysiological recording and $[Ca^{2+}]_i$ measurement respectively. The islet cells were cultured at 37 °C in RPMI-1640 medium at 7 mM glucose supplemented with 10% heat-inactivated FCS, 100 IU/ml penicillin, and 100 μ g/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 . The culture medium was changed every 2 days. The cells were used for the experiments during days 3–6 in culture.

Insulin secretion assay

After culture overnight, the islets were washed twice with cold incubating solution and then separated into 48-well plates with 20 islets per well in 0.5 ml incubating solution. The samples were randomly separated into different groups with eight samples per group. To observe the dose effects of GW9508, the incubating solution was collected after incubating with different treatments for 1 h at stationary state. To observe the effects of GW9508 on the first and second phases of GSIS, 200 islets per sample were put into perfusion chamber and perfused at a speed of 0.1 ml/min under the control of a peristaltic pump. Each experiment began after a 20-min equilibrium period of perfusion with perfusion solution containing 2 mM glucose. Then the perfusion solution was collected every 5 min. After being perfused at 2 mM glucose to get the basal insulin secretion, the islets were exposed to 15 mM glucose for 60 min and then washed with 2 mM glucose. GW9508 was put into the perfusion solution 10 min before the exposure to 15 mM glucose. The perfusion and incubating solution was composed of the following (mM): 140 NaCl, 4.7 KCl, 2.6 $CaCl_2$, 1.2 $MgSO_4$, 1 $NaHCO_3$, 1.2 Na_2HPO_4 , 2 glucose, and 5 HEPES (pH=7.4 with NaOH). Insulin levels in the incubating solution were assayed by insulin ELISA kit as indicated by the kit instructions (DSL Laboratory, Webster, TX, USA). In brief, samples were pipetted into an ELISA 96-well plates in duplicate. The anti-insulin conjugate antibodies were then added, and the plates were incubated on a plate shaker at 700 r.p.m. for 2 h at room temperature. Then the plates were washed thoroughly and 3,3',5,5'-tetramethylbenzidine substrate solution was added and incubated for 15 min at room temperature in the dark. Stop buffer was added and then the absorbance was measured at 450 nm.

Figure 1

Effects of GW9508 on glucose-stimulated insulin secretion. Glucose at 5 and 15 mM significantly stimulated insulin secretion compared with 2 mM glucose. GW9508 at 20 and 40 μM but not at 10 μM significantly inhibited insulin secretion stimulated by 5 and 15 mM glucose. GW9508 did not influence insulin secretion under 2 mM glucose (* $P < 0.05$ and ** $P < 0.01$ vs control at same glucose levels, $n = 8$).

with a MultiSkan ELISA plate reader. At the end of the collection of perfusion solution and the incubating solution, the islets in each sample were centrifuged and the suspension solution was removed. Total DNA from the samples was extracted using QIAamp DNA Investigator kits and was quantified using Qubit dsDNA HS assay kits with Qubit 2.0 Fluorometer as indicated by the kit instructions. The insulin level was rectified by the total DNA quantity for each sample.

Electrophysiological recording in single rat β-cells

Recordings were made using perforated whole-cell patch clamp configuration. Electrodes were pulled by a Sutter P-87 microelectrode puller from borosilicate micro-pipettes and had an initial input resistance of 3–5 MΩ. All recordings were performed using the Axopatch 200A amplifier (Axon Instrument, Union City, CA, USA). The bath solution for membrane potential recording and K_{ATP} current recording was same to the insulin assay. The pipette solution for membrane potential and K_{ATP} current recording was composed of the following (mM): 76 K_2SO_4 , 10 KCl, 10 NaCl, 8 $MgSO_4$, and 20 HEPES (pH = 7.3 with KOH). Membrane perforation was achieved by nystatin (final concentration 0.24 mg/ml in pipette solution). After formation of a high-resistance seal, the voltage in the pipette was held at -80 mV. The series resistance reaching lower than 30 MΩ was considered as full access to the cell interior. The whole-cell capacitance and series resistance were well compensated and then the membrane potential

or K_{ATP} current was recorded using different protocols. Membrane potential was recorded under current-clamp mode using Axoscope 8 program, as the current was held at 0 pA. K_{ATP} current was recorded under voltage-clamp mode using Clampex 8 program. The cells were held at -80 mV, and a trial of sweeps was obtained by clamping from -130 to -30 mV at intervals of 1 s with 20 mV increments and a duration of 300 ms each step. Pancreatic β-cells were identified by cell size and cell membrane capacitance. The mean capacitance of β- and α-cells are 5.5 ± 0.3 and 2.8 ± 0.1 pF respectively (Gopel *et al.* 2000, Leung *et al.* 2005). For this study, the cells whose whole-cell capacitances were above 6 pF were recorded and the data were used for analysis. Experiments were performed at room temperature.

Measurement of $[Ca^{2+}]_i$ in single rat β-cells

Islet 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 20 min in this solution to allow full de-esterification of the dye and tranquilization of the cells. Fura-2 in the cell was alternately excited by 340 and 380 nm light (100 ms exposure and 2-s cycle) under the control of Dual-Wavelength Photometry Controller (SDR, NSW, Australia). Emission at 510 nm was detected by a photomultiplier tube (PMT model 77348, Oriel, Bozeman, MT, USA). The signal was transmitted into DigiData and recorded by

Figure 2

Effects of GW9508 on the first and second phases of glucose-stimulated insulin secretion. Insulin secretion was significantly increased in a biphasic pattern under 15 mM glucose stimulation. GW9508 treatment 10 min before exposure to 15 mM glucose completely inhibited the first phase of insulin secretion and significantly inhibited the second phase of insulin secretion (* $P < 0.01$ vs control at the same time point, $n = 5$).

Axoscope 8.2 (Axon Instrument). $[Ca^{2+}]_i$ was calculated according to the formula described by Grynkiewicz *et al.* (1985). $[Ca^{2+}]_i$ (nM) = $K_d \times (F_0/F_s) \times (R - R_{min}) / (R_{max} - R)$, where K_d , the Fura-2 disassociation constant (225 nM); F_0 , the 380 nm fluorescence in the absence of Ca^{2+} ; F_s , the 380 nm fluorescence with saturating Ca^{2+} ; R , the 340:380 nm fluorescence ratio; R_{max} , the 340:380 nm ratio with saturating Ca^{2+} ; and R_{min} , the 340:380 nm ratio in the absence of Ca^{2+} . F_0/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 Ca^{2+} 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 Ca^{2+} with 8 mM EGTA. 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 the perfusion. The bath solution used for $[Ca^{2+}]_i$ measurements was the same as that used for the insulin assay.

Statistical analysis

The data are represented as mean $\pm$ S.E.M. for each group. One-way ANOVA was used to analyse the statistical significance between different groups. $P < 0.05$ was taken as the minimum level of statistical significance.

Results

GW9508 dose dependently inhibited GSIS from rat islets

Glucose significantly stimulated insulin secretion from rat pancreatic islets at 5 and 15 mM concentrations during 1-h incubation compared with the basal level of 2 mM glucose. GW9508 did not significantly influence insulin secretion at 2 mM glucose. However, it dose dependently inhibited GSIS during 1-h incubation. GW9508 at 10 μ M did not influence insulin secretion at either 5 or 15 mM glucose levels. At 5 mM glucose, GW9508 at 20 and 40 μ M significantly inhibited insulin secretion 33 and 64% respectively (4.77 ± 0.65 ng/ μ g islets DNA in control; 3.2 ± 0.39 ng/ μ g islets DNA at 20 μ M GW9508, $P < 0.05$ vs control, $n = 8$; 1.71 ± 0.31 ng/ μ g islets DNA at 40 μ M GW9508, $P < 0.01$ vs control, $n = 8$). At 15 mM glucose, GW9508 at 20 and 40 μ M significantly inhibited insulin secretion 19 and 51% respectively (13.66 ± 0.74 ng/ μ g islets DNA in control; 11.11 ± 0.49 ng/ μ g islets DNA at 20 μ M GW9508, $P < 0.05$ vs control, $n = 8$; 6.67 ± 0.76 ng/ μ g islets DNA at 40 μ M GW9508, $P < 0.01$ vs control, $n = 8$; Fig. 1). Glucose stimulated two phases of

insulin secretion in rat islets as shown by the perfusion experiment. When GW9508 was given 10 min before the exposure to 15 mM glucose, GW9508 (40 μ M) completely inhibited the first phase of GSIS. The second phase of GSIS was also significantly inhibited by GW9508, and the inhibitory ratio was about 51% (Fig. 2). GW9508 at 20 and 40 μ M could not inhibit insulin secretion that was stimulated by 0.1 mM tolbutamide at 15 mM glucose during 1-h incubation (Fig. 3).

GW9508 induced hyperpolarization and activated K_{ATP} channels in rat β -cells

The resting membrane potential of rat β -cells was -48 ± 2 mV at 5 mM glucose and was significantly hyperpolarized to -66 ± 2 mV by 40 μ M GW9508 ($P < 0.01$, $n = 8$). The cells recovered from the hyperpolarization fully in 5 min after washout of GW9508 (Fig. 4A and B). GW9508 (40 μ M) immediately increased K_{ATP} currents from -4 ± 0.7 to -16 ± 2 pA/pF at -130 mV ($P < 0.01$, $n = 8$). The current recovered completely to -4 ± 0.5 pA/pF, 5 min after washout of GW9508 (Fig. 4C). Tolbutamide decreased K_{ATP} current from -4 ± 0.5 to -2 ± 0.3 pA/pF at -130 mV ($P < 0.01$, $n = 5$). GW9508 (40 μ M), up to 10 min, did not affect this current in the presence of tolbutamide (Fig. 4D). The dose-responses of GW9508 on membrane potential and K_{ATP} currents were shown in Fig. 4E and F. At 5 mM glucose, GW9508 at 10 μ M did not affect the membrane potential and K_{ATP} currents, and GW9508 at 20 and 40 μ M significantly induced hyperpolarization of membrane potential and increase in K_{ATP} currents dose dependently.

Figure 3

Effects of GW9508 on tolbutamide-stimulated insulin secretion. 0.1 mM tolbutamide stimulated high levels of insulin secretion, and GW9508 at 20 and 40 μ M could not inhibit tolbutamide-stimulated insulin secretion.

Figure 4

Effects of GW9508 on K_{ATP} channels in rat β -cells. (A) GW9508 at 40 μ M induced hyperpolarization of membrane potential at 5 mM glucose with a full recovery. (B) The statistical analysis of the membrane potential (** $P < 0.01$ vs control, $n = 8$). (C) GW9508 significantly increased K_{ATP} current with full recovery after removal of GW9508 (** $P < 0.01$ vs control at same voltage level, $n = 8$). (D) 0.1 mM tolbutamide significantly reduced K_{ATP} current, and GW9508 could not increase the current after addition of tolbutamide. (E) GW9508 dose dependently stimulated hyperpolarization of membrane potential (* $P < 0.05$ and ** $P < 0.01$ vs control, $n = 5$). (F) GW9508 dose dependently increased K_{ATP} currents (** $P < 0.01$ vs control and ## $P < 0.01$ vs control and 20 μ M GW9508, $n = 5$).

Rat pancreatic β -cells depolarized and generated action potentials when the membrane potential reached about -40 mV in response to 15 mM glucose, and similar responses were repetitively observed after glucose stimulation (Fig. 5A). The resting membrane potential of rat β -cells was significantly depolarized by 15 mM glucose (Fig. 5B, -47 ± 3 vs -28 ± 7 , $P < 0.05$, $n = 6$). GW9508 (40 μ M) acutely induced hyperpolarization of membrane potential, and 15 mM glucose could not induce depolarization in the presence of GW9508. Addition of 0.1 mM tolbutamide, however, evoked depolarization of membrane potential in the presence of GW9508 (Fig. 5C). The statistical result of the membrane potential before and after GW9508 is shown in Fig. 5D.

GW9508 inhibited glucose-stimulated increase in $[Ca^{2+}]_i$ in rat β -cells

The resting $[Ca^{2+}]_i$ level in β -cells was low at 3 mM glucose, but it significantly increased after glucose stimulation. Glucose repetitively induced increase in $[Ca^{2+}]_i$ in rat β -cells (Fig. 6A). The second increase in $[Ca^{2+}]_i$ was analysed and the $[Ca^{2+}]_i$ levels were shown in Fig. 6B (15 ± 2 vs 115 ± 13 nM, $P < 0.01$, $n = 7$). GW9508 (40 μ M) treatment completely blocked 15 mM glucose-stimulated increase in $[Ca^{2+}]_i$ but not 0.1 mM tolbutamide-stimulated increase in $[Ca^{2+}]_i$ in rat β -cells (Fig. 6C). The $[Ca^{2+}]_i$ levels in the second stimulation were analysed and shown in Fig. 6D (18 ± 3 nM at basal level, 20 ± 2 nM after 15 mM glucose, $P > 0.5$ vs basal level; 126 ± 17 nM after tolbutamide, $P < 0.01$ vs basal level, $n = 7$).

GW9508 stimulated increase in $[Ca^{2+}]_i$ by mobilizing calcium release in rat β -cells

GW9508 itself stimulated a transient increase in $[Ca^{2+}]_i$ in rat β -cells (Fig. 7A). Removal of extracellular Ca^{2+} did not eliminate the GW9508-stimulated increase (Fig. 7B). Pretreatment of the cells with thapsigargin (1 μ M for 30 min) totally eliminated the GW9508-stimulated $[Ca^{2+}]_i$ increase (Fig. 7C). The mean $[Ca^{2+}]_i$ changes ($\Delta[Ca^{2+}]_i$, the mean $[Ca^{2+}]_i$ levels in 3 min after GW9508 stimulation subtracting basal levels of $[Ca^{2+}]_i$) are shown in Fig. 7D ($P < 0.01$ vs control in thapsigargin group, $n = 8$).

Figure 5

Effects of GW9508 on glucose-induced depolarization of β -cells. (A) The membrane potential of rat pancreatic β -cells depolarized with generation of action potentials in response to 15 mM glucose repetitively. (B) The statistical analysis of the membrane potential in the second stimulation with glucose (* $P < 0.05$, $n = 6$). (C) GW9508 blocked 15 mM glucose-induced depolarization but not tolbutamide-stimulated depolarization. (D) The statistical analysis of the membrane potential in the second time treatment by GW9508, glucose, and tolbutamide (** $P < 0.01$ vs other three groups, $n = 6$).

Figure 6

Effects of GW9508 on glucose-induced increase in $[Ca^{2+}]_i$ in rat β -cells. (A) 15 mM glucose induced the increase in $[Ca^{2+}]_i$ in rat β -cells. (B) The statistical analysis of the maximal levels of $[Ca^{2+}]_i$ stimulated by glucose (** $P < 0.01$ vs 3 mM glucose, $n = 7$). (C) GW9508 blocked glucose-induced increase in $[Ca^{2+}]_i$, but not tolbutamide-stimulated increase in $[Ca^{2+}]_i$ in rat β -cells. (D) The statistical analysis of the maximal levels of $[Ca^{2+}]_i$ stimulated by glucose and tolbutamide (no significant difference between 3 and 15 mM glucose; ** $P < 0.01$ vs 3 and 15 mM glucose, $n = 7$).

Effects of U73122 treatment on GW9508-induced activation of K_{ATP} channels and increase in $[Ca^{2+}]_i$ in β -cells

Activation of phospholipase C (PLC) and the subsequent production of inositol triphosphate (IP₃) is one of the signalling pathways for ligand-stimulated calcium release from intracellular calcium stores (Briscoe *et al.* 2003, Fujiwara *et al.* 2005). We tested the effect of PLC blockade by U73122 on GW9508-induced activation of K_{ATP} channels and increase in $[Ca^{2+}]_i$ in β -cells. The K_{ATP} currents were activated to $567 \pm 85\%$ of basal levels by 40 μ M GW9508. After U73122 treatment (10 μ M for 10 min), the K_{ATP} currents were activated to $250 \pm 51\%$ of basal levels by 40 μ M GW9508 (Fig. 8A, $P < 0.01$ vs control, $n = 8$). The $[Ca^{2+}]_i$ levels increased significantly by GW9508 stimulation, and this increase was totally blocked by U73122 treatment (10 μ M for 10 min). The mean level of GW9508-stimulated $[Ca^{2+}]_i$ was $287 \pm 65\%$ of the control, which decreased to $108 \pm 12\%$ of the control in U73122 group (Fig. 8B, $P < 0.01$ vs non-U73122 control group, $n = 8$).

Discussion

GW9508 was first identified as FFAR agonist to activate GPR40 and GPR120 (Briscoe *et al.* 2006, Sum *et al.* 2007, Tikhonova *et al.* 2007). In the present study, we found

that GW9508 inhibits GSIS by activating K_{ATP} channels in rat pancreatic β -cells, which is supported by the results of insulin secretion assay, electrophysiological recording, and $[Ca^{2+}]_i$ recording.

Glucose stimulates insulin secretion in a biphasic manner that is mediated by two different signalling pathways, the K_{ATP} channel-dependent pathway and K_{ATP} channel-independent pathway (Gembal *et al.* 1992, Komatsu *et al.* 1997, Straub *et al.* 1998, Henquin 2000, Aizawa *et al.* 2002). ATP derived from glucose oxidation closes K_{ATP} channels and leads to depolarization of membrane potential and subsequent activation of voltage-gated Ca^{2+} channels ($Ca^{2+}_{(v)}$ channels). The increase in $[Ca^{2+}]_i$ following the influx of Ca^{2+} via $Ca^{2+}_{(v)}$ channels triggers the first phase of insulin secretion in K_{ATP} channel-dependent manner (Rorsman 1997). Augmentation of Ca^{2+} -triggered insulin secretion by K_{ATP} channels independent of signals that follow glucose metabolism takes part in the second phase of insulin secretion (Straub *et al.* 1998, Straub & Sharp 2002). GW9508 completely inhibited the first phase of GSIS, and it partially inhibited the second phase of GSIS. It supports that GW9508 mainly inhibits the K_{ATP} -dependent pathway but not K_{ATP} -independent pathway.

Pancreatic K_{ATP} channels are formed by two different types of subunits, the Kir6.2 inwardly rectifying potassium channels and sulphonylurea receptor subunit SUR1. Pancreatic K_{ATP} channels are specifically blocked by sulphonylurea such as tolbutamide. Although GW9508

Figure 7

Effects of GW9508 on $[Ca^{2+}]_i$ levels in rat β -cells. (A) GW9508 stimulated transient increase in $[Ca^{2+}]_i$ in rat β -cells in normal solution. (B) Removal of extracellular Ca^{2+} did not eliminate GW9508-stimulated increase in $[Ca^{2+}]_i$. (C) Thapsigargin treatment (1 μ M for 30 min) fully eliminated GW9508-stimulated increase in $[Ca^{2+}]_i$. (D) Analysis of the mean $[Ca^{2+}]_i$ changes (** $P < 0.01$ vs control, $n = 8$).

Figure 8

Effects of PLC inhibition on GW9508-induced activation of K_{ATP} channels and increase in $[Ca^{2+}]_i$ in rat β -cells. (A) U73122 treatment (10 μ M for 10 min) partly inhibited the activation of K_{ATP} currents induced by GW9508 (** $P < 0.01$ vs control, $n = 8$). (B) U73122 treatment completely blocked the increase in $[Ca^{2+}]_i$ stimulated by GW9508 (** $P < 0.01$ vs control, $n = 8$). The dotted lines indicate the 100% of basal levels.

inhibited GSIS, it could not inhibit tolbutamide-stimulated insulin secretion. This supports that GW9508 inhibits GSIS by opening K_{ATP} channels. The subsequent electrophysiological recording showed that GW9508 induced hyperpolarization of membrane potential and increase in K_{ATP} currents, giving strong evidence that GW9508 activated K_{ATP} channels. $[Ca^{2+}]_i$ recording further confirmed that GW9508 inhibited GSIS by opening K_{ATP} channels because the increase in $[Ca^{2+}]_i$ stimulated by glucose but not by tolbutamide was inhibited GW9508.

Although GW9508 mobilizes intracellular calcium stores, the present study suggests that the effect is not strong enough to stimulate insulin secretion and to counteract its inhibitory effects on GSIS. GW9508-stimulated increase in $[Ca^{2+}]_i$ was eliminated by depletion of intracellular endoplasmic reticulum calcium stores by

thapsigargin treatment, but not by removal of extracellular calcium. It is therefore suggested that GW9508 stimulates calcium release from intracellular calcium stores. Previous reports suggested that FFAR such as GPR40 activation is linked to PLC and mobilization of intracellular calcium stores by production of IP3 (Briscoe *et al.* 2003, Fujiwara *et al.* 2005). Moreover, it has been confirmed that GPR40 activation can stimulate phosphatidylinositol hydrolysis, further supporting that the IP3 signalling pathway is employed by GPR40 (Meidute Abaraviciene *et al.* 2008). The present results indicate that GW9508 may activate GPR40 and subsequently stimulate calcium release from intracellular calcium stores. This is further confirmed by the observation that inhibition of PLC activity by U73122 totally blocked GW9508-stimulated increase in $[Ca^{2+}]_i$ in rat β -cells. However, this increase in $[Ca^{2+}]_i$ is not sufficient enough to stimulate insulin secretion as there was no significant increase in insulin secretion after GW9508 stimulation at 2 mM glucose and no augmenting effect was observed of GW9508 on tolbutamide-stimulated insulin secretion. As the increase in $[Ca^{2+}]_i$ stimulated by GW9508 is transient and comes from release of intracellular Ca^{2+} stores, it may not be sufficient in either a temporal or spatial manner to stimulate exocytosis of insulin granules. On the other hand, the process of exocytosis can be regulated by membrane potential, and it is indicated that depolarization can enhance exocytosis voltage dependently (Mochida *et al.* 1998). Hyperpolarization of the membrane potential by GW9508 may counteract the stimulatory effects of $[Ca^{2+}]_i$ increase on exocytosis of insulin granules.

GW9508 mobilized intracellular calcium by activating PLC, but the effects of GW9508 on K_{ATP} channels were not solely mediated by PLC. Blockade of PLC by U73122 only inhibited the activation of K_{ATP} channels by GW9508 in part, suggesting that other signalling pathways may also be involved. It has been reported that oleic acid promotes the activation of the extracellular signal-regulated protein kinase–MAPK signalling pathway, mainly via FFAR (Zhang *et al.* 2007). This indicates that multiple signalling pathways are employed by FFAR. In our previous study, we also tried to clarify the signalling molecules that are involved in the activation of K_{ATP} channels by GW9508, but it was found that neither protein kinase A nor C is involved in the action of GW9508 on K_{ATP} channels (Zhao *et al.* 2008). Further investigation will be required to clarify the candidate signals in mediating the activation of K_{ATP} channels by GW9508 and the involvement of FFAR.

Previous reports mostly indicate that FFAR activation by FFAs stimulates insulin secretion (Itoh *et al.* 2003, Fujiwara *et al.* 2005, Salehi *et al.* 2005, Shapiro *et al.* 2005). While it was reported that GW9508 is an agonist of FFAR, the inhibitory effects of GW9508 on GSIS are controversial to the stimulatory effects of FFAs on insulin secretion. The discrepancy may be due to differences in species and the significant physiological difference between cell lines and islets β -cell in primary culture. Briscoe *et al.* (2006) did not see the stimulatory effects of 10 μ M GW9508 on insulin secretion in mouse or rat islets, although they observed the stimulatory effects of GW9508 on insulin secretion in MIN6 cells. On the other hand, FFAs not only act as ligands to activate FFAR but also enter cells to be metabolized. The metabolic effects of FFAs could not be fully excluded in previous reports about the stimulation of insulin secretion by FFAs through FFAR. GW9508 does not generate intracellular metabolic effects by itself upon activation of FFAR (Briscoe *et al.* 2006, Sum *et al.* 2007). The different chemical properties between FFAs and GW9508 may be one reason for the discrepancy in action on insulin secretion. Moreover, the possibility that GW9508 activates K_{ATP} channels via a non-FFAR signalling pathway has not been ruled out. This possibility can be clarified by observing the effects of GW9508 on insulin secretion from islets of FFAR knockout animals such as GPR40 knockout mice. Owing to the limitation to obtaining the animal models, we at present are not able to exclude the possibility of non-FFAR effects of GW9508. However, our results suggest that it is worthy to observe the effects of GW9508 in GPR40 knockout mice, which would deepen our comprehension of the mechanism of GW9508-induced activation of K_{ATP} channels.

In summary, the present study suggests that GW9508 is not a secretagogue for insulin secretion in rat islets. GW9508 above 20 μ M inhibits insulin secretion via activating K_{ATP} channels. Although PLC is involved in the action of GW9508 on K_{ATP} channels, the detailed signalling pathways for the effects of GW9508 needs to be further investigated.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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