Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 335 (2005) 97–104
BBRC
www.elsevier.com/locate/ybbrc

Role of GPR40 in fatty acid action on the β cell line INS-1E ☆

Hagit Shapiro [a], Sigal Shachar [a], Israel Sekler [b], Michal Hershfinkel [c], Michael D. Walker [a,*]

[a] Department of Biological Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel
[b] Department of Physiology, Faculty of Health Science, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel
[c] Department of Morphology, Faculty of Health Science, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

Received 6 July 2005

Abstract

GPR40 is a G protein-coupled receptor expressed preferentially in β cells, that has been implicated in mediating free fatty acid-stimulated insulin release. GPR40 RNAi impaired the ability of palmitic acid (PA) to increase both insulin secretion and intracellular calcium ($[Ca^{2+}]_i$). The PA-dependent $[Ca^{2+}]_i$ increase was attenuated by inhibitors of Gαq, PLC, and SERCA. Thus GPR40 activates the Gαq pathway, leading to release of Ca^{2+} from the ER. Yet the GPR40-dependent $[Ca^{2+}]_i$ rise was dependent on extracellular Ca^{2+} and elevated glucose, and was blocked by inhibition of L-type calcium channels (LTCC) or opening of the K_{ATP} channel; this suggests that GPR40 promotes Ca^{2+} influx through up-regulation of LTCC pre-activated by glucose and membrane depolarization. Taken together, the data indicate that GPR40 mediates the increase in $[Ca^{2+}]_i$ and insulin secretion through the Gαq–PLC pathway, resulting in release of Ca^{2+} from the ER and leading to up-regulation of Ca^{2+} influx via LTCC.

Keywords: Diabetes; Fatty acid; GPR40; Insulin; Pancreas

Insulin secretion from pancreatic β cells is primarily mediated by glucose and augmented by other nutrients, hormones, and neural stimuli [1]. Elevated concentrations of glucose lead to intracellular metabolism of the sugar, generation of ATP, closure of ATP-regulated K^+ (K_{ATP}) channels, membrane depolarization, and opening of voltage-dependent L-type Ca^{2+} channels (LTCC). The resulting increase in intracellular calcium ($[Ca^{2+}]_i$) serves as the triggering signal leading to secretion of insulin [1,2]. Free fatty acids (FFA) and other nutrients stimulate glucose-induced insulin release in vitro [3,4], and in vivo they increase plasma insulin levels [5,6]. In β cells, FFA serve as secondary stimuli to glucose because they are ineffective alone, but increase insulin release in the presence of elevated glucose [1]. Since FFA can enter the β cell and be activated into long chain fatty-acyl CoA (LC-CoA), exposure of β cells to FFA such as palmitic acid (PA) elevates the intracellular levels of LC-CoAs [4]. High glucose levels reduce oxidation of PA in β cells because malonyl-CoA, which is generated by glucose metabolism in the mitochondria, inhibits carnitine-palmitoyl-transferase1 (CPT-1). Therefore, exposure of β cells to high glucose and PA causes elevation of LC-CoA which has been proposed to have multiple intracellular effects including modulation of protein kinase C (PKC), acylation of proteins, and direct activation of insulin exocytosis [4,7,8]. In addition, PA together

☆ *Abbreviations:* BSA, bovine serum albumin; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CPA, cyclopiazonic acid; ER, endoplasmic reticulum; FFA, free fatty acid; GPR40, G protein-coupled receptor 40; $InsP_3$, inositol-1,4,5-triphosphate; K_{ATP}, ATP-regulated K^+ channel; LC-CoA, long chain fatty-acyl CoA; LTCC, L-type calcium channel; PA, palmitic acid; PTX, pertussis toxin; PKC, protein kinase C; PLC, phospholipase C; RS, RETRO-SUPER; RNAi, RNA interference; SERCA, sarco-endoplasmic reticulum Ca^{2+}-ATPase; SOCC, store-operated calcium channels.

* Corresponding author. Fax: +972 8 934 4118.
E-mail address: m.walker@weizmann.ac.il (M.D. Walker).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.07.042

with high glucose increase the intracellular ATP/ADP ratio and increase the $[Ca^{2+}]_i$ levels [3,7].

Long chain FFA can activate GPR40, a G protein-coupled receptor expressed preferentially in pancreatic β cells and in insulin-secreting β cell lines [9–11]. A wide range of saturated fatty acids from C12 to C18 and unsaturated fatty acids from C18 to C22 are able to increase $[Ca^{2+}]_i$ in non-β cells ectopically expressing GPR40 with an EC_{50} of 10–100 μM. Fatty acid addition to the pancreatic β cell line MIN6 induced an increase in $[Ca^{2+}]_i$ and glucose-stimulated insulin secretion, and GPR40 down-regulation inhibited the ability of fatty acids to increase glucose-stimulated insulin exocytosis [10]. However, the signaling pathways linking the activation of GPR40 to insulin secretion have not been identified [8,12].

In this study, we analyze the function of GPR40 in the β cell line INS-1E. We demonstrate that reduction of GPR40 gene expression by retrovirus-mediated RNAi methodology leads to loss of ability of PA to increase $[Ca^{2+}]_i$ levels and insulin secretion. Using fluorescent measurement of $[Ca^{2+}]_i$ levels in combination with pharmacological inhibitors, we reveal the mechanism of GPR40 signaling in INS-1E cells. The GPR40-mediated increase in $[Ca^{2+}]_i$ levels is dependent on the activation of Gαq–phospholipase C (PLC) pathway and release of Ca^{2+} from the ER, leading to up-regulation of calcium influx to β cells through LTCC.

Materials and methods

Materials. All reagents were purchased from Sigma except pertussis toxin (Calbiochem), BI (LC Laboratories), and YM-254890 (gift of Dr. M. Taniguchi from Astellas Pharma Inc.). PA (100 mM stock solution) was dissolved in 100 mM NaOH by heating at 70 °C in a shaking bath according to [13]. Stock solutions were diluted in assay buffer to obtain the final concentration as indicated.

Cell culture. Rat insulinoma INS-1E cells [14] were cultured in RPMI 1640 medium including 10% fetal calf serum and additions were as previously described [15]. Measurement of insulin secretion and intracellular calcium was performed using INS-1E cells between passages 52 and 89.

Stable RNA interference clones. Stable RNA interference (RNAi) clones against rat GPR40 (EMBL/GenBank/DDBJ Accession No. AF539810) were made using the pRETRO-SUPER (RS) retroviral vector system (gift from Dr. R. Agami, The Netherlands Cancer Institute) [16,17]. To generate pRETRO-SUPER-RNAi40, pRETRO-SUPER vector was digested with *Bgl*II and *Hin*dIII, and annealed oligos (5′gatccccACTGCGACTCACCCCCAGCttcaagagGCTGGGGGTGAGTCGCAGTtttttggaaa3′ and 5′agcttttccaaaaaACTGCGACTCACCCCCAGCtctcttgaaGCTGGGGGTGAGTCGCAGTggg3′) were ligated into the vector. The 19 nt GPR40 target sequences are indicated in upper case letters. Ecotropic retroviral supernatants were generated from this vector and control pRETRO-SUPER vector by transfection of HEK 293T cells using calcium phosphate precipitation. After 48 h, the media of the transfected cells were filtered (0.45 μm) and used for infection of INS-1E cells [16] in the presence of 4 μg/ml polybrene. The infected cells were selected using 2 μg/ml puromycin. The RNAi40 and control (RS) stable lines were cultured for one passage and then used to prepare RNA to determine GPR40 gene expression.

RNA preparation and real-time PCR. RNA was extracted from INS-1E cells using the TRI reagent kit (Molecular Research Center). Synthesis of cDNA was performed using 2 μg RNA in the presence of 200 U/μl Superscript II RT (Invitrogen) and 10 μM random hexamer primers (Roche). Real-time PCRs were carried out using 20 ng cDNA, 200 nM oligos corresponding to rat GPR40 or rat cyclophilin, and 10 μl SYBR-Green PCR master Mix (ABI). Rat GPR40 oligos were, 5′CCCTGCCCGACTCAGTTTC3′ and 5′GGCAGCCCACATAGCAGAA3′; rat cyclophilin oligos were, 5′TCACCATCTCCGACTGTGGA3′ and 5′AAATGCCCGCAAGTCAAAGA3′. The PCRs were performed in an ABI 7000 sequence detection system. Expression of GPR40 was normalized to that of cyclophilin.

Insulin secretion measurements. INS-1E cells were cultured for 2–4 passages after infection, then seeded at a density of 7×10^5 in 60-mm plates and cultured for 3 days. Cells were incubated at 37 °C for 2 h in glucose-free RPMI, washed twice with glucose-free RPMI, and incubated for 30 min at 37 °C in glucose-free Krebs–Ringer bicarbonate Hepes buffer (KRBH) containing 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 2 mM $NaHCO_3$, 10 mM Hepes, pH 7.4, and 0.1% bovine serum albumin (fatty acid free). Cells were then washed once with glucose-free KRBH and incubated for 30 min at 37 °C in KRBH containing the indicated stimuli. After 30 min, supernatants from each plate were collected and the cellular insulin content was determined using acid–ethanol extraction [15]. Insulin concentration in media and in acid–ethanol extracts was measured by radioimmunoassay (Linco, St. Louis) using rat insulin as a standard and rat insulin specific antibody. Insulin secreted is expressed as a percentage of cellular insulin content.

Fluorescent calcium imaging. INS-1E cells were seeded at a density of 3×10^5 in 24-well plates containing coverslips. After 3–5 days, cells were loaded for 30 min at 22 °C with 1 μM Fura-2 AM (Molecular probes, Eugene, OR) in glucose-free loading buffer (138 mM NaCl, 5.6 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 10 mM Hepes, pH 7.35) [18]. The Fura-2 solution was removed and cells were incubated with loading buffer for a further 30 min at 22 °C. Coverslips were mounted in a perfusion chamber placed on a microscope stage and Ca^{2+} imaging was carried as described [19]. The imaging system consisted of a Zeiss Axiovert 100 inverted microscope, Polychrome II monochromator (T.I.L.L Photonics, Germany), and a cooled CCD (PCO). Fluorescent imaging measurements were acquired using Axon Imaging Workbench 2, and the response was analyzed using Excel. The 340/380 fluorescence ratio was determined from at least three experiments (40–50 cells per experiment), and processed using a macro that permits peak synchronization and determines maximal peak height. Means ± SEM of stimulation index were calculated from peak height minus the peak height before stimuli, relative to cells treated with 2.5 mM glucose, which was defined as 1. The fluorescence ratio of one representative cell is shown for each experiment in Results section, and in the Supplementary figures section.

Results

In order to examine the role of GPR40 in β cells, we used the clonal rat cell line INS-1E, which has been shown to display glucose- and fatty acid-dependent insulin secretion [20]. GPR40 mRNA levels in INS-1E cells were reduced using a retroviral expression system encoding interfering RNA (RNAi) specific for rat GPR40; INS-1E cells expressing RNAi against GPR40 (RNAi40) showed 78% reduction in mRNA level compared to cells containing pRETRO-SUPER vector

(RS), as analyzed by quantitative real-time PCR (Fig. 1A). In response to 25 mM glucose, insulin secretion was stimulated to a similar extent in both cell lines; addition of PA (25 μM) led to a further stimulation of insulin secretion in control cells, but no increase in RNAi40 cells (Fig. 1B). Intracellular calcium levels were estimated in INS-1E cells using the Ca^{2+} indicator, Fura-2. Increasing glucose concentrations from 2.5 to 25 mM led to a $[Ca^{2+}]_i$ rise in both RS and RNAi40 cells; addition of PA led to a further increase in $[Ca^{2+}]_i$ in RS cells, but little or no increase in RNAi40 cells (Fig. 1C). Thus, the PA effect on $[Ca^{2+}]_i$ and insulin secretion depends on GPR40.

Since GPR40 increases $[Ca^{2+}]_i$ levels in β cells, we next carried out a series of experiments to characterize the mechanism of GPR40 signaling in INS-1E cells by measuring $[Ca^{2+}]_i$ levels. The GPR40 effect on intracellular Ca^{2+} levels was dependent on the presence of elevated concentrations of glucose (Figs. 2A and C). Incubation with 22.5 mM sorbitol and 2.5 mM glucose produced no significant increase in $[Ca^{2+}]_i$ levels (data not shown), indicating that the $[Ca^{2+}]_i$ elevation is not due to an osmotic effect.

Since glucose is known to promote the influx of extracellular Ca^{2+}, we tested whether extracellular Ca^{2+} was required for GPR40 signaling (Figs. 2B and C). We observed that GPR40 signaling was abolished in the absence of extracellular Ca^{2+}. This requirement for extracellular Ca^{2+} may reflect an intrinsic property of the intracellular GPR40 signaling pathway; for example a component of the GPR40 signaling cascade may be a Ca^{2+}-dependent enzyme. Alternatively, GPR40 may stimulate Ca^{2+} influx in a glucose-dependent manner; thus GRP40 may act on LTCC which have been pre-activated (“primed”) by glucose. The experiment presented in Fig. 2 distinguishes between these possibilities since incubation of cells with glucose in the presence of extracellular calcium followed by addition of PA, in the absence of calcium, led to complete loss of the GPR40 response (Fig. 2). We therefore suggest that GPR40 signaling requires influx of Ca^{2+}, and is likely to involve stimulation of LTCC which were pre-activated by glucose, resulting in increased $[Ca^{2+}]_i$ and leading to augmented insulin secretion.

When incubations were performed in the presence of nimodipine, an inhibitor of the LTCC, or diazoxide, an opener of the K_{ATP} channel, the PA effect was sharply re-

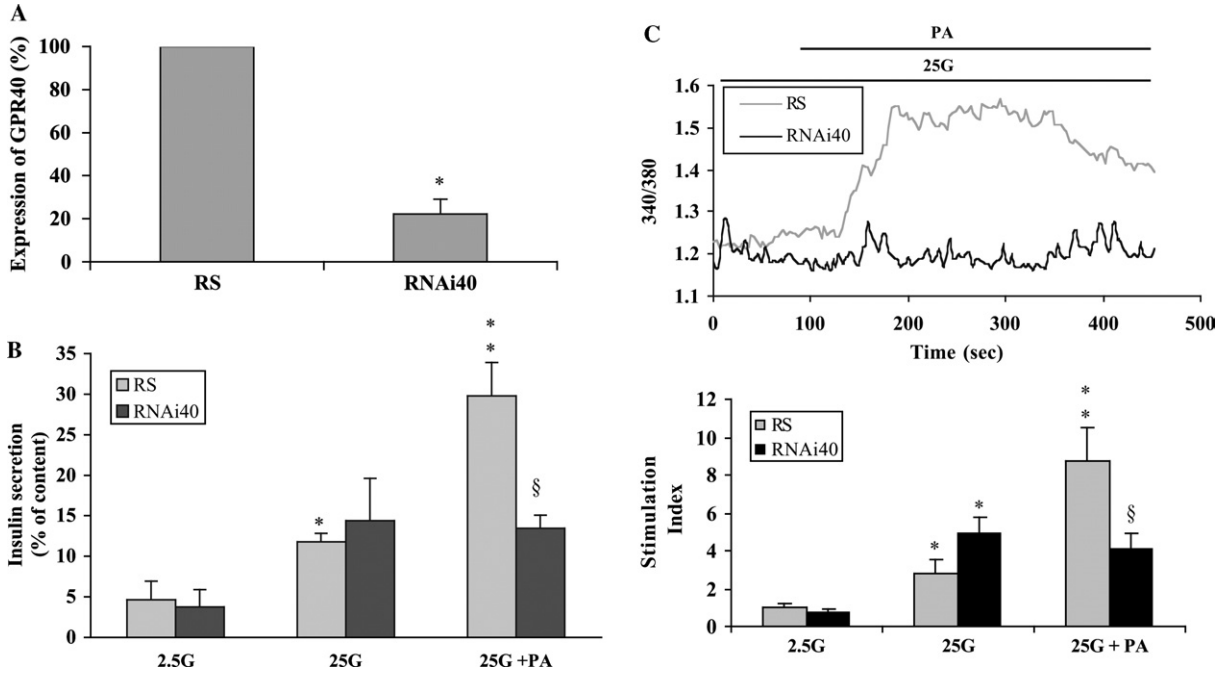

Fig. 1. Effect of RNAi40 on PA-dependent insulin secretion and intracellular calcium. Stably transformed cell lines were derived by infection of INS-1E cells with control retrovirus pRETRO-SUPER (RS) or pRETRO-SUPER RNAi-GPR40 (RNAi40). (A) Relative expression of GPR40 mRNA in RS and RNAi40 cells was measured by real-time PCR as described under Materials and methods. Results are presented as means ± SEM ($n = 4$). *$P < 0.01$. (B) Effect of RNAi40 on PA-dependent insulin secretion. RS and RNAi40 cells were exposed to 2.5 mM glucose (2.5G), 25 mM glucose (25G) or 25 mM glucose in the presence of 25 μM PA (25G + PA). Secretion of insulin was determined as described under Materials and methods. *$P < 0.05$ for 2.5G vs. 25G tested in RS cells; **$P < 0.05$ for 25G vs. 25G + PA tested in RS cells; $^{§}P = 0.02$ in RNAi40 vs. RS cells treated with 25G + PA. (C) Effect of RNAi40 on PA-dependent intracellular calcium levels. RS and RNAi40 cells were exposed to 25 mM glucose (25G). At the time indicated, 25 μM PA (PA) was added. Intracellular calcium levels were determined as described under Materials and methods. The upper panel represents 340/380 fluorescence ratio of a representative cell. The lower panel shows the stimulation index (mean ± SEM) determined as described under Materials and methods. The stimulation index of RNAi40 cells is expressed relative to RS cells treated with 2.5G, defined as 1. *$P < 0.05$ for 2.5G vs. 25G tested in RS or RNAi40 cells; **$P < 0.05$ for 25G vs. 25G + PA tested in RS cells; $^{§}P < 0.05$ in RS vs. RNAi40 cells treated with 25G + PA.

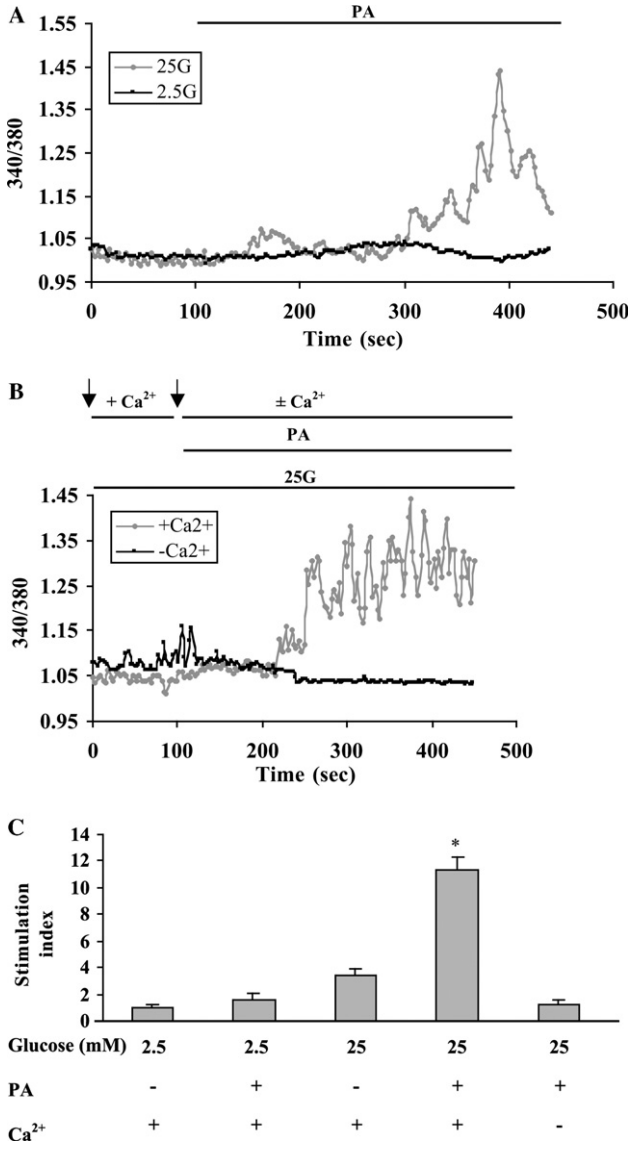

Fig. 2. Effect of low glucose levels or omission of extracellular Ca^{2+} on PA-stimulated $[Ca^{2+}]_i$. (A) INS-1E cells were exposed to 2.5 mM glucose (2.5G) or 25 mM glucose (25G), then 25 μM PA was added. The traces show 340/380 fluorescence ratio of a representative cell. (B) INS-1E cells were incubated in the presence of 25 mM glucose (25G) and 2.5 mM Ca^{2+}, then 25 μM PA was added in the presence of 25 mM glucose, or 25 mM glucose in buffer lacking Ca^{2+}. The traces show fluorescence ratio (340/380) of a representative cell. (C) Summary of experiments shown in A and B: stimulation index of $[Ca^{2+}]_i$ (mean ± SEM). $^*P < 0.01$ for 25G + PA, vs. 25G, vs. 2.5G + PA, and vs. 25G + PA − Ca^{2+}.

duced (Fig. 3 and Supplementary Fig. 1). This confirms that the GPR40-stimulated increase in $[Ca^{2+}]_i$ depends on glucose-mediated activation of K_{ATP} channels and LTCC.

We next studied the intracellular pathway activated by GPR40 leading to increased $[Ca^{2+}]_i$. Incubation of INS-1E cells with pertussis toxin (PTX, 100 ng/ml), an inhibitor of Gαi, did not significantly affect the GPR40 response (Supplementary Fig. 2). On the other hand, the Gαq inhibitor, YM-254890 [21], significantly re-

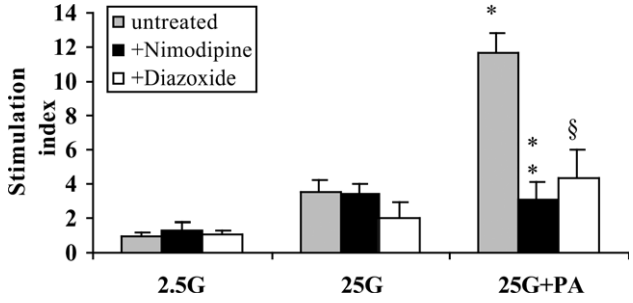

Fig. 3. Effect of LTCC inhibition and K_{ATP} channel opening on PA-stimulated $[Ca^{2+}]_i$. Stimulation index of $[Ca^{2+}]_i$ (mean ± SEM) of INS-1E cells incubated with 25 mM glucose (25G), then treated with 25 μM PA + 5 μM nimodipine (LTCC inhibitor) or 250 μM diazoxide (K_{ATP} channel opener). $^*P < 0.01$ for 25G vs. 25G + PA; $^{**}P < 0.01$ for 25G + PA vs. 25G + PA + nimodipine; $^{\S}P < 0.01$ for 25G + PA vs. 25G + PA + diazoxide.

duced the GPR40-induced Ca^{2+} rise (Fig. 4A). As a positive control, we verified that this inhibitor significantly reduced the increase in $[Ca^{2+}]_i$ mediated by UTP (Fig. 4A, inset [22]). Thus the GPR40 response involves activation of the Gαq pathway. Application of the PLC inhibitor, U73122, (2 μM) also significantly reduced the response (Fig. 4B), whereas treatment with U73343, an inactive analogue, had little effect (Fig. 4B). Activation of PLC leads to production of inositol-1,4,5-triphosphate ($InsP_3$) which causes release of Ca^{2+} from internal stores like the ER. To test the importance of Ca^{2+} release from ER stores, we used cyclopiazonic acid (CPA), an inhibitor of the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA). As expected, CPA (50 μM) caused a large increase in $[Ca^{2+}]_i$ (not shown), as a result of Ca^{2+} emptying from intracellular stores; following this treatment, the ability of the cells to respond to PA was significantly reduced (Fig. 4C), indicating that the GPR40 response first involves release of Ca^{2+} from ER stores. These findings suggest that GPR40 activation by PA leads to stimulation of the Gαq–PLC pathway. Thus, GPR40 function in β cells involves both a metabotropic pathway activating the Gαq–PLC leading to release of Ca^{2+} from ER stores and an ionotropic pathway up-regulating calcium transport through LTCC.

The release of Ca^{2+} from intracellular Ca^{2+} stores may trigger the opening of store-operated calcium channels (SOCC) [23], leading to an ionotropic increase in $[Ca^{2+}]_i$. Trivalent cations such as La^{3+} and Gd^{3+} have been reported to effectively inhibit SOCC [24], but application of La^{3+} (2 μM) did not affect the increase in $[Ca^{2+}]_i$ caused by GPR40 activation (data not shown). This indicates that the ionotropic component of the Ca^{2+} response triggered by GPR40 does not involve SOCC activation, but rather activation of LTCC. Taken together, our data suggest that the GPR40-stimulated increase in insulin secretion depends on a Gαq pathway involving PLC, emptying of ER stores, and leading to up-regulation of LTCC.

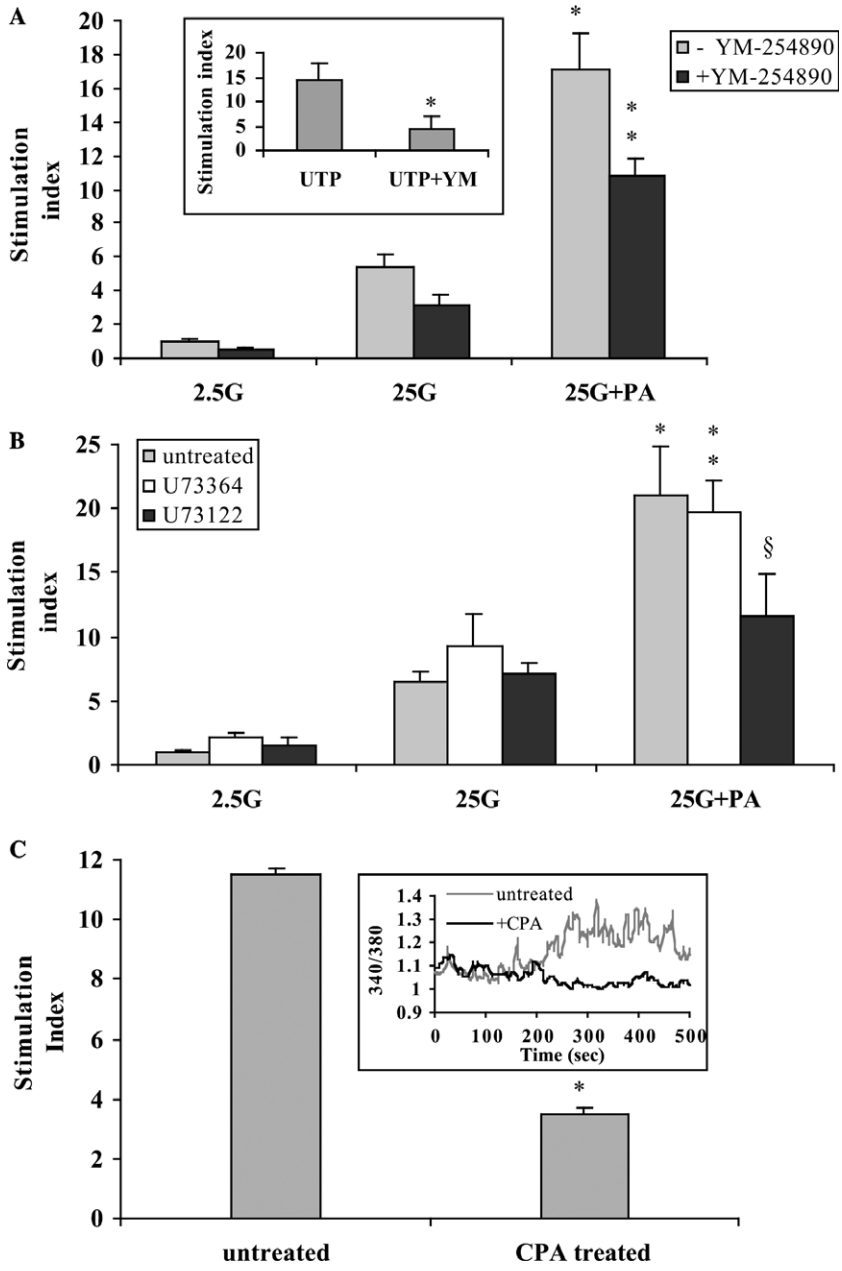

Fig. 4. Effect of Gαq inhibition, PLC inhibition, and emptying ER stores on PA-stimulated $[Ca^{2+}]_i$. (A) Stimulation index of $[Ca^{2+}]_i$ (mean ± SEM) of INS-1E cells incubated for 5 min in the presence or absence of 1 μM YM-254890, then treated with 25 μM PA ± YM-254890. $^*P < 0.01$ for 25G vs. 25G + PA; $^{**}P < 0.05$ for 25G + PA vs. 25G + PA + YM-254890. Inset, stimulation index of INS-1E cells treated with 100 μM UTP and then incubated for 5 min with UTP + YM-254890. $^*P < 0.05$ for UTP vs. UTP + YM-254890. (B) Stimulation index of $[Ca^{2+}]_i$ (mean ± SEM) for cells incubated in medium containing 25 mM glucose and then treated with 25 μM PA ± 2 μM U73122 or U73364. $^*P < 0.01$ for 25G vs. 25G + PA; $^{**}P < 0.05$ for 25G vs. 25G + PA + U73364; $^{\S}P < 0.05$ for 25G + PA vs. 25G + PA + U73122. (C) Stimulation index of $[Ca^{2+}]_i$ (mean ± SEM) for cells incubated with 25 mM glucose in the presence or absence of 50 μM CPA (SERCA inhibitor). Following return of $[Ca^{2+}]_i$ to basal levels after CPA treatment, 25 μM PA was added in buffer lacking CPA. $^*P < 0.01$ for 25G + PA tested in cells treated with CPA vs. control cells. Inset, fluorescence ratio (340/380) of a representative cell.

Discussion

Long chain fatty acids have important effects on pancreatic β cell function; acute treatment augments glucose-dependent insulin secretion, whereas chronic exposure leads to deterioration of β cell function (lipotoxicity). The mechanism underlying these actions is not well understood. The G protein-coupled receptor GPR40 has been reported to be selectively expressed in pancreatic β cells, and to be activated by long chain fatty acids [9–11]. The phenotypes of mice over-expressing or lacking GPR40 indicate an important role for GPR40 in both acute and chronic actions of fatty acids on β cells [25]. However, the mechanism by which

GPR40 mediates its actions on β cells has not been established.

In this study, we examined the role of GPR40 in mediating acute effects of fatty acids in the pancreatic β cell line INS-1E. We show that RNAi-mediated reduction of GPR40 expression caused significant reduction in insulin secretion, consistent with results obtained previously with the β cell line MIN6 [10]. Using the Ca^{2+} indicator Fura-2, we show that the ability of PA to increase $[Ca^{2+}]_i$ in β cells is GPR40 dependent. This experimental system was used to analyze the signaling pathway activated by GPR40 action in β cells. We demonstrate that the GPR40-mediated $[Ca^{2+}]_i$ increase depends on the presence of elevated glucose, membrane depolarization, and activation of LTCC. But the glucose signal is not sufficient for the GPR40 response since β cells pre-incubated with high glucose and Ca^{2+}, then treated with PA and high glucose in the absence of Ca^{2+}, completely fail to show an increase in $[Ca^{2+}]_i$. Thus GPR40 signaling requires extracellular Ca^{2+} and pre-activation of LTCC by glucose, leading to further stimulation of LTCC mediated by GPR40.

Use of selective inhibitors revealed that the activation of GPR40 by PA leads to stimulation of the Gαq-dependent PLC signaling pathway, and involves mobilization of calcium from ER stores (Fig. 5). We suggest that although Ca^{2+} release from intracellular stores is necessary, the major $[Ca^{2+}]_i$ rise is mediated by the subsequent up-regulation of Ca^{2+} influx resulting from stimulation of LTCC, consistent with the recent finding that PA increases β cell L-type Ca^{2+} currents [26]. We conclude that GPR40-mediated PA action in β cells activates the Gαq–PLC pathway, causing release of ER Ca^{2+}, and leading to stimulation of LTCC (Fig. 5).

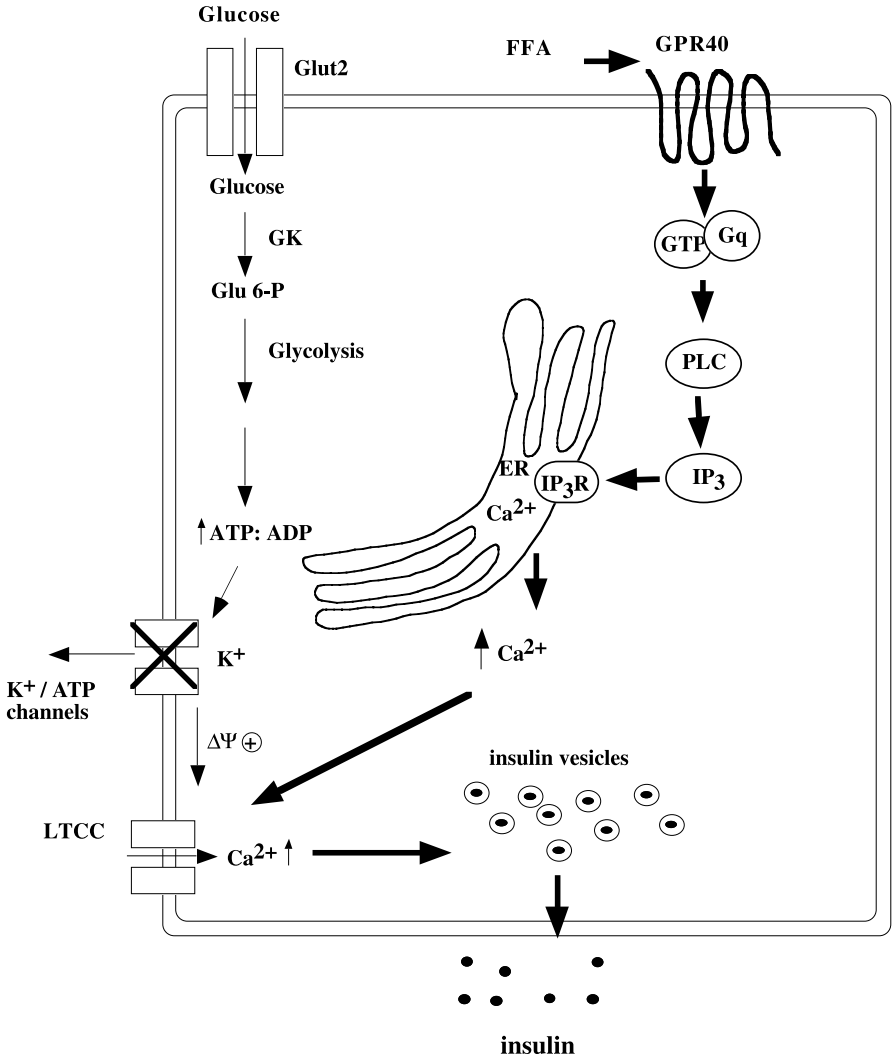

Fig. 5. Proposed mechanism for GPR40 action in β cells. GPR40 activation by PA stimulates the Gαq–PLC signaling pathway leading to Ca^{2+} release from ER stores. In the presence of extracellular Ca^{2+} and elevated glucose this leads to stimulation of LTCC, resulting in further increase in $[Ca^{2+}]_i$ and insulin release. Bold arrows indicate the suggested mechanism activated by GPR40. Also shown is the pathway for glucose-stimulated insulin secretion in β cells. $IP_3 = InsP_3$.

In this study, we employed the saturated fatty acid PA, since saturated fatty acids have been reported to be more effective insulin secretagogues than unsaturated fatty acids [27], and PA is the most abundant fatty acid in plasma [28] and one of the most potent agonists of rat GPR40 [10]. We show for the first time the GPR40 signaling pathway induced by PA. In parallel to this study, Fujiwara et al. [29] have very recently reported that the ability of the unsaturated fatty acid, oleic acid, to stimulate $[Ca^{2+}]_i$ in β cells is mediated by GPR40 and blocked by inhibitors of LTCC or PLC. Consistent with the results of Fujiwara et al., our results show that the GPR40 response produced by PA depends on extracellular calcium and permeation through LTCC. Importantly, we show that opening of the K_{ATP} channel, using diazoxide, blocks the stimulatory effect of GPR40 on the LTCC. This indicates that PA-mediated GPR40 signaling requires membrane depolarization leading to LTCC activation. Thus GPR40 signaling is not sufficient for LTCC opening, but acts on pre-activated LTCC, thereby increasing Ca^{2+} permeation into β cells. We also show directly for the first time that the GPR40 response activates the Gαq pathway, using the Gαq inhibitor YM-254890. In contrast to Fujiwara et al., we show that emptying the ER Ca^{2+} stores significantly reduces the GPR40 response. Therefore, our results indicate that PA-mediated GPR40 activates a metabotropic signaling pathway involving Gαq, PLC, and release of Ca^{2+} from the ER which leads to an ionotropic pathway involving activation of LTCC (Fig. 5). Gαq-dependent regulation of LTCC has recently been demonstrated in frog oocytes where it is mediated via PKC [30], and in myocytes where it is mediated by intracellular NO release [31]. In preliminary experiments, we did not see an effect of inhibitors of PKC, PI3 kinase or ERK on the GPR40 activation of LTCC (data not shown). Hence, the specific pathway involved in the cross-talk between release of ER Ca^{2+} and LTCC activation remains to be identified. A role for the G protein βγ subunit in mediating the action of GPR40 seems less likely considering the inhibitory effect of CPA on the Ca^{2+} response [32].

The actions of FFA on β cells have generally been considered to be mediated through intracellular lipid-derived metabolites such as LC-CoA that have been proposed to affect multiple signaling pathways, leading to increased insulin secretion [4,7]. Our results demonstrate that knock-down of GPR40 strongly inhibits both increased intracellular Ca^{2+} and insulin release, indicating that FFA may modify β cell function directly through GPR40, independent of intracellular LC-CoA. This conclusion is consistent with a recent report demonstrating that the actions of PA on insulin exocytosis in mouse islets are not mimicked by intracellular application of palmitoyl CoA [26].

PA, like other β cell secretagogues, has little or no effect in the presence of low concentrations of glucose [1], a property important to avoid hypoglycemia. Our data show that the GPR40-dependent activation of LTCC is ineffective unless the Ca^{2+} channel has been primed by pre-exposure to glucose. An important goal for the future will be the identification of the link between GPR40, intracellular calcium, and glucose signaling pathways in the normal and diabetic states.

Acknowledgments

This study was supported in part by grants to M.D.W. from the European Union, the Israel Academy of Sciences and Humanities, the Roy and Ellen Rosenthal Family Foundation, and the Laufer Trust. M.D.W. is the incumbent of the Marvin Meyer and Jenny Cyker Chair of Diabetes Research. We thank Dr. C. Wollheim, Dr. R. Seger, and Dr. Z. Vogel for valuable discussions, Dr. C. Wollheim and Dr. P. Maechler for the gift of INS-1E cells, and Dr. R. Agami for providing the pRET-RO-SUPER vector. The YM-254890 was a gift from Dr. M. Taniguchi, Astellas Pharma Inc., Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2005.07.042.

References

[1] J.C. Henquin, M.A. Ravier, M. Nenquin, J.C. Jonas, P. Gilon, Hierarchy of the beta-cell signals controlling insulin secretion, Eur. J. Clin. Invest. 33 (2003) 742–750.
[2] S.G. Straub, G.W. Sharp, Glucose-stimulated signaling pathways in biphasic insulin secretion, Diab. Metab. Res. Rev. 18 (2002) 451–463.
[3] C. Warnotte, P. Gilon, M. Nenquin, J.C. Henquin, Mechanisms of the stimulation of insulin release by saturated fatty acids. A study of palmitate effects in mouse beta-cells, Diabetes 43 (1994) 703–711.
[4] M. Prentki, S. Vischer, M.C. Glennon, R. Regazzi, J.T. Deeney, B.E. Corkey, Malonyl-CoA and long chain acyl-CoA esters as metabolic coupling factors in nutrient-induced insulin secretion, J. Biol. Chem. 267 (1992) 5802–5810.
[5] L.L. Madison, W.A. Seyffert Jr., R.H. Unger, B. Barker, Effect on plasma free fatty acids on plasma glucagon and serum insulin concentrations, Metabolism 17 (1968) 301–304.
[6] S.R. Crespin, W.B. Greenough 3rd, D. Steinberg, Stimulation of insulin secretion by infusion of free fatty acids, J. Clin. Invest. 48 (1969) 1934–1943.
[7] B.E. Corkey, J.T. Deeney, G.C. Yaney, K. Tornheim, M. Prentki, The role of long-chain fatty acyl-CoA esters in beta-cell signal transduction, J. Nutr. 130 (2000) 299S–304S.
[8] V. Poitout, The ins and outs of fatty acids on the pancreatic beta cell, Trends Endocrinol. Metab. 14 (2003) 201–203.

[9] C.P. Briscoe, M. Tadayyon, J.L. Andrews, W.G. Benson, J.K. Chambers, M.M. Eilert, C. Ellis, N.A. Elshourbagy, A.S. Goetz, D.T. Minnick, P.R. Murdock, H.R. Sauls Jr., U. Shabon, L.D. Spinage, J.C. Strum, P.G. Szekeres, K.B. Tan, J.M. Way, D.M. Ignar, S. Wilson, A.I. Muir, The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids, J. Biol. Chem. 278 (2003) 11303–11311.

[10] Y. Itoh, Y. Kawamata, M. Harada, M. Kobayashi, R. Fujii, S. Fukusumi, K. Ogi, M. Hosoya, Y. Tanaka, H. Uejima, H. Tanaka, M. Maruyama, R. Satoh, S. Okubo, H. Kizawa, H. Komatsu, F. Matsumura, Y. Noguchi, T. Shinohara, S. Hinuma, Y. Fujisawa, M. Fujino, Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40, Nature 422 (2003) 173–176.

[11] K. Kotarsky, N.E. Nilsson, E. Flodgren, C. Owman, B. Olde, A human cell surface receptor activated by free fatty acids and thiazolidinedione drugs, Biochem. Biophys. Res. Commun. 301 (2003) 406–410.

[12] G.A. Rutter, Insulin secretion: fatty acid signalling via serpentine receptors, Curr. Biol. 13 (2003) R403–R405.

[13] S.P. Cousin, S.R. Hugl, C.E. Wrede, H. Kajio, M.G. Myers Jr., C.J. Rhodes, Free fatty acid-induced inhibition of glucose and insulin-like growth factor I-induced deoxyribonucleic acid synthesis in the pancreatic beta-cell line INS-1, Endocrinology 142 (2001) 229–240.

[14] A. Merglen, S. Theander, B. Rubi, G. Chaffard, C.B. Wollheim, P. Maechler, Glucose sensitivity and metabolism-secretion coupling studied during two-year continuous culture in INS-1E insulinoma cells, Endocrinology 145 (2004) 667–678.

[15] M. Asfari, D. Janjic, P. Meda, G. Li, P.A. Halban, C.B. Wollheim, Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines, Endocrinology 130 (1992) 167–178.

[16] T.R. Brummelkamp, R. Bernards, R. Agami, Stable suppression of tumorigenicity by virus-mediated RNA interference. A system for stable expression of short interfering RNAs in mammalian cells, Cancer Cell 2 (2002) 243–247.

[17] T.R. Brummelkamp, R. Bernards, R. Agami, A system for stable expression of short interfering RNAs in mammalian cells, Science 296 (2002) 550–553.

[18] G. Kang, G.G. Holz, Amplification of exocytosis by Ca^{2+}-induced Ca^{2+} release in INS-1 pancreatic beta cells, J. Physiol. 546 (2003) 175–189.

[19] M. Hershfinkel, A. Moran, N. Grossman, I. Sekler, A zinc-sensing receptor triggers the release of intracellular Ca^{2+} and regulates ion transport, Proc. Natl. Acad. Sci. USA 98 (2001) 11749–11754.

[20] B. Rubi, P.A. Antinozzi, L. Herrero, H. Ishihara, G. Asins, D. Serra, C.B. Wollheim, P. Maechler, F.G. Hegardt, Adenovirus-mediated overexpression of liver carnitine palmitoyltransferase I in INS1E cells: effects on cell metabolism and insulin secretion, Biochem. J. 364 (2002) 219–226.

[21] M. Taniguchi, K. Suzumura, K. Nagai, T. Kawasaki, J. Takasaki, M. Sekiguchi, Y. Moritani, T. Saito, K. Hayashi, S. Fujita, S. Tsukamoto, K. Suzuki, YM-254890 analogues, novel cyclic depsipeptides with Galpha(q/11) inhibitory activity from *Chromobacterium* sp. QS3666, Bioorg. Med. Chem. 12 (2004) 3125–3133.

[22] A.D. Conigrave, L. Jiang, Review: Ca(2+)-mobilizing receptors for ATP and UTP, Cell Calcium 17 (1995) 111–119.

[23] K. Kiselyov, X. Xu, G. Mozhayeva, T. Kuo, I. Pessah, G. Mignery, X. Zhu, L. Birnbaumer, S. Muallem, Functional interaction between InsP3 receptors and store-operated Htrp3 channels, Nature 396 (1998) 478–482.

[24] A. Gore, A. Moran, M. Hershfinkel, I. Sekler, Inhibitory mechanism of store-operated Ca^{2+} channels by zinc, J. Biol. Chem. 279 (2004) 11106–11111.

[25] P. Steneberg, N. Rubins, R. Bartoov-Shifman, M.D. Walker, H. Edlund, The FFA receptor GPR40 links hyperinsulinemia, hepatic steatosis and impaired glucose homeostasis in mouse, Cell Metab. 1 (2005) 245–258.

[26] C.S. Olofsson, A. Salehi, C. Holm, P. Rorsman, Palmitate increases L-type Ca^{2+} currents and the size of the readily releasable granule pool in mouse pancreatic beta-cells, J. Physiol. 557 (2004) 935–948.

[27] C. Warnotte, M. Nenquin, J.C. Henquin, Unbound rather than total concentration and saturation rather than unsaturation determine the potency of fatty acids on insulin secretion, Mol. Cell. Endocrinol. 153 (1999) 147–153.

[28] A.J. Brown, S. Jupe, C.P. Briscoe, A family of fatty acid binding receptors, DNA Cell Biol. 24 (2005) 54–61.

[29] K. Fujiwara, F. Maekawa, T. Yada, Oleic acid interacts with GPR40 to induce Ca^{2+} signaling in rat islet beta-cells: mediation by phospholipase C- and L-type Ca^{2+} channel and link to insulin release, Am. J. Physiol. Endocrinol. Metab. (2005), published online May 25, 2005.

[30] S. Weiss, T. Doan, K.E. Bernstein, N. Dascal, Modulation of cardiac Ca^{2+} channel by Gq-activating neurotransmitters reconstituted in *Xenopus* oocytes, J. Biol. Chem. 279 (2004) 12503–12510.

[31] Y.G. Wang, E.N. Dedkova, X. Ji, L.A. Blatter, S.L. Lipsius, Phenylephrine acts via IP3-dependent intracellular NO release to stimulate L-type Ca^{2+} current in cat atrial myocytes, J. Physiol. (2005), published online June 9, 2005.

[32] J. Zhong, J.R. Hume, K.D. Keef, Beta-adrenergic receptor stimulation of L-type Ca^{2+} channels in rabbit portal vein myocytes involves both alphas and betagamma G protein subunits, J. Physiol. 531 (2001) 105–115.