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Free Fatty Acid Receptor 1 (FFA₁R/GPR40) and Its Involvement in FA Stimulated Insulin Secretion

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ABSTRACT

Free fatty acids have generally been proposed to regulate pancreatic insulin release by an intracellular mechanism involving inhibition of CPT-1. The recently de-orphanized G-protein coupled receptor, FFA₁R/GPR40, has been shown to be essential for fatty acid stimulated insulin release in MIN6 mouse insulinoma cells. The CPT-1 inhibitor, 2-bromo palmitate (2BrP), was investigated for its ability to interact with mouse FFA₁R/GPR40. It was found to inhibit IP₃ hydrolysis induced by linoleic acid (LA) (100 μ M in all experiments) in HEK293 cells transfected with FFA₁R/GPR40 and in the MIN6 subclone, MIN6c4. 2BrP also inhibited LA-stimulated insulin release from mouse pancreatic islets. Mouse islets were subjected to antisense intervention by treatment with a FFA₁R/GPR40 specific morpholino oligonucleotide for 48 h. Antisense treatment of islets suppressed LA-stimulated insulin release by 50% and by almost 100% when islets were pretreated with LA for 30 min before applying the antisense. Antisense treatment had no effect on tolbutamide-stimulated insulin release. Confocal microscopy using a FFA₁R/GPR40 specific antibody revealed receptor expression largely localized to the plasma membrane of insulin producing cells. Pretreating the islets with LA for 30 min followed by antisense oligonucleotide treatment for 48 h reduced the FFA₁R/GPR40 immunoreactivity to background levels. The results demonstrate that FFA₁R/GPR40 is inhibited by the CPT-1 inhibitor, 2BrP, and confirm that FFA₁R/GPR40 is indeed necessary, at least in part, for fatty acid stimulated insulin release.

INTRODUCTION

Glucose and free fatty acids (FFAs) are the two most important regulators of insulin release. Medium-chain to long-chain fatty acids (FAs) are known to have pleiotropic effects on the pancreatic β -cell; while acute administration of FFAs stimulates insulin release (Haber et al. 2003), long-term exposure to high levels of FFAs results in impairment of the β -cell secretory capacity (Salehi et al. 2003, Salehi et al. 2001, Bollheimer et al. 1998), often associated with type 2 diabetes. The secretory response is strongly dependent on the metabolic status of the β -cell. Thus, in fasting rodents or humans, FFAs are essential to permit basal insulin secretion and to allow a secretory response to glucose on termination of the fasting period (Dobbins et al. 1998a). However, the mechanism by which FFAs potentiate insulin secretion is not fully understood. It has been generally proposed that, in order to exert a stimulatory effect on insulin release, FFAs need to enter the cell and conjugate with coenzyme A forming long-chain acyl-CoA (LC-CoA) (Corkey et al. 2000). According to the same proposed mechanism LC-CoA then serves as a major effector molecule in the β -cell influencing a multitude of signaling pathways, including protein kinase C and the exocytotic machinery (Corkey et al. 2000).

Recently, we identified the orphan G-protein coupled receptor (GPCR) previously known as GPR40 as a receptor of FFAs, and we tentatively named it FFA₁R (Kotarsky et al. 2003b). The receptor couples to G-proteins of both the G _{α q} and to the pertussis toxin sensitive G _{α i} type, affecting several signaling pathways. GPR40 was “de-orphanized” simultaneously by another two groups (Briscoe et al. 2003, Itoh et al. 2003). The receptor is activated by medium- to long-chain FFAs and, quite notably, we also found that it responded to antidiabetic drugs of the thiazolidinedione type (Kotarsky et al. 2003b). All of the original three reports (Kotarsky et al.

2003b, Briscoe et al. 2003, Itoh et al. 2003) agree that a carbon chain of at least 10 atoms and a free carbonyl group seem to be major structural requirements for receptor activation. However, the conclusions concerning the degree of saturation differed. While our results (Kotarsky et al. 2003b) indicated a slight preference for saturated FAs, the reports of Briscoe *et al.* (Briscoe et al. 2003) and Itoh *et al.* (Itoh et al. 2003) showed no differences between saturated and unsaturated FAs. The FFA₁R/GPR40 transcript was found preferentially in β -cells and in mouse insulinoma MIN6 cells, and also in monocytes (Kotarsky et al. 2003b, Briscoe et al. 2003, Itoh et al. 2003). We also observed that activation of the receptor resulted in an increase in cytosolic $[Ca^{2+}]$. Since an increase in cytosolic calcium concentration is a prerequisite for insulin release, this observation strongly indicated that FFA₁R/GPR40 activation is an important, if not necessary, component in this process. Our results were confirmed by Itoh *et al.* (Itoh et al. 2003), showing FFA₁R/GPR40, indeed, to be necessary for FFA potentiation of insulin release in MIN6 cells.

The halogenated fatty acid, 2-bromo palmitate (2BrP), originally described as an inhibitor of carnitine palmitoyl transferase 1 (CPT-1), has been shown to either potentiate glucose stimulated insulin release (GSIS) (Prentki et al. 1992, Chen et al. 1994) or inhibit GSIS (Warnotte et al. 1994, Parker et al. 2003). The scope of the present study was to provide evidence that FA activation and 2BrP inhibition both modulate insulin release by interaction with FFA₁R/GPR40, not only in MIN6 cells but also in intact mouse pancreatic islets. The results obtained confirm earlier indications and show that FFA₁R/GPR40 activation in intact mouse islets is, indeed, necessary for the FA-mediated potentiation of GSIS (Itoh et al. 2003).

MATERIAL AND METHODS

Animals

Female mice of the NMRI strain (B&K, Sollentuna, Sweden), weighing 28-32g, were used throughout the experiments. They were given a standard pellet diet (B&K) and tap water *ad libitum*. All animals used for preparation of pancreatic islets were killed by cervical dislocation. The experimental procedures were approved by the local Ethical Committee for Animal Research.

Drugs and Chemicals

Collagenase and HRP-conjugated goat anti-rabbit IgG were from Sigma and Pierce Biotechnology (Rockford, IL), respectively. Cy2-conjugated anti-mouse IgG and Cy5-conjugated anti-guinea pig IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA). Guinea pig-raised antiinsulin antibody was from Eurodiagnostica (Malmö, Sweden). FA-free bovine serum albumin (BSA) was from Boehringer Mannheim. Triacsin C was from BIOMOL (Plymouth Meeting, PA). FA-free BSA was from Roche. All other chemicals were from Merck or Sigma. Stock solutions of linoleic acid (LA) and 2BrP were made in 95% ethanol. Test substances were prepared in solution containing 1.0% (w/v) FA-free BSA, as previously described by Spector *et al* (Spector et al. 1971). Before the experiments the stock solution was dissolved in the appropriate culture or incubation medium to achieve the desired concentrations of test substances.

Cell culture and transfection

A subclone of the MIN6 cell line, MIN6c4, was grown in Dulbecco's modified Eagle's medium (DMEM) with Glutamax-1 (Invitrogen, Paisley, UK) supplemented with 15% heat-inactivated FBS (Invitrogen), 60 μ M β -mercaptoethanol, 50 units/ml penicillin and 50 μ g/ml streptomycin.

HeLa (Kotarsky et al. 2003a) and HEK293 cells were grown in DMEM with Glutamax-1 supplemented with 3% FBS, 50 units/ml penicillin and 50 µg/ml streptomycin. All cells were maintained in a 37 °C incubator with 7% CO₂. The mouse GPR40 ORF (Genbank accession number AB095745) was amplified with PCR (forward primer, 5' GCCAAGCTTACCATGGACCTGCCCCACAGCTCTCCTTCG 3'; reverse primer, 5' GGCGAATTCCTACTTCTGAATTGTTCTCTTTGAGTC 3'), subcloned into the pEAK12 expression vector (Edge BioSystems, Gaithersburg, MD), and then transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Total time of transfection was 6 h and the cells were assayed 48-72 h later.

PI-hydrolysis

Receptor activation was assayed by measuring phosphatidyl inositol (PI) hydrolysis essentially as described by Kang and Leeb-Lundberg (Kang and Leeb-Lundberg 2002). Briefly, MIN6c4 cells were pre-loaded with myo-[³H]inositol (Perkin Elmer, Boston, MA) for 16-20 h and stimulated with LA, 2BrP, and/or tolbutamide for 30 min. The cells were lysed with formic acid on ice and the inositol phosphates were isolated using anion exchange chromatography. For ease of comparison, the results from repeated PI hydrolysis measurements were normalised against the mean basal hydrolysis activity of untreated cells (set to 1) and expressed as "relative PI hydrolysis". The coefficient of variation (interassay differences) was 11% for the transfected HEK293 cells and 7% for MIN6c4 cells.

Insulin secretion from isolated islets

Preparation of pancreatic islets from mice was performed by retrograde injection of a collagenase solution via the bile-pancreatic duct. Islets were then isolated and hand-picked under a stereomicroscope at room temperature (Gotoh et al. 1985). Thereafter, the islets were preincubated for 30 min at 37 °C in Krebs-Ringer bicarbonate buffer, pH 7.4, supplemented with 10 mM HEPES, 1% FA-free BSA and 1 mM glucose. After preincubation the buffer was changed and the islets were incubated at 8.3 mM glucose with 1% FA-free BSA, in the absence or presence of different test agents, for 60 min at 37 °C. In experiments including triacsin C, it was present at either 1 or 10 µM during both preincubation and incubation. Each incubation vial contained 8-12 islets in 1 ml of buffer solution and was gassed with 95% O₂-5% CO₂ to obtain a constant pH and oxygenation. All incubations were performed in a shaking incubator at 30 cycles/min. An aliquot of the medium was removed immediately after incubation and frozen for later assay of insulin. The radioimmunoassay kit used for insulin determination was obtained from Diagnostika (Falkenberg, Sweden). In experiments involving antisense intervention, islets were cultured for 48 h in RPMI 1640 (SVA, Uppsala, Sweden) supplemented with 10% calf serum, 100 units/ml penicillin, 10 µg/ml streptomycin in the presence or absence of a mFFA₁R/mGPR40 specific morpholino antisense oligonucleotide, M40 (Fig. 1) (Heasman 2002) (Gene Tools, Philomath, OR) at a concentration of 1.4 µM. A non-specific random-sequence morpholino was used as control. Prior to addition to the incubation medium, the morpholinos were complexed with the manufacturer's special delivery system according to the instructions. To generate single β-cells, the freshly isolated islets were shaken at low extracellular Ca²⁺ as previously described (Olofsson et al. 2004). The dispersed β-cells were then plated on a plastic

petri dish (Nunc, Denmark) and maintained in culture for the same time period as for the islets (48 h).

Confocal microscopy

All incubations of freshly isolated islets were performed as above. The islets were then fixed in 4% formaldehyde and permeabilized with 5% Triton X-100. Mouse FFA₁R/GPR40 was detected with a rabbit polyclonal antibody (1:100), raised against the C-terminal peptide, NH₂-CVTRTQRGTIQK-COOH, in combination with Cy2-conjugated anti-rabbit IgG (1:150).

Nonspecific binding was blocked with 5% Normal Donkey Serum (Jackson ImmunoResearch Laboratories). For staining of insulin, islets were incubated with the antiinsulin antibody (1:1000) followed by an incubation with a Cy5-conjugated anti-guinea pig IgG antibody (1:150). The fluorescence was visualized in a Pascal LSM510 confocal microscope by sequential scanning at the excitation/emission wavelengths 488/505-530nm (Cy2) or 633/>650nm (Cy5).

Statistics

The data are calculated as means \pm SEM. The level of significance between groups was assessed using unpaired Student's t-test or analysis of variance followed by Tukey-Kramer's test whenever appropriate. In the figures, *** = $p < 0.001$ and ** = $p < 0.01$.

RESULTS

2-Bromo palmitate (2BrP) inhibits mFFA₁R/GPR40 in transfected HEK293 cells

The well described CPT-1 inhibitor, 2BrP (Chen et al. 1994, Chase and Tubbs 1972), has been reported also to inhibit FA-mediated potentiation of insulin release (Parker et al. 2003, Warnotte et al. 1994). In order to test if 2BrP also has effects on the FFA₁R/GPR40, the mouse receptor was transiently expressed in HEK293 cells and challenged with linoleic acid (LA), 2BrP or with a mixture of both. LA (100 μ M) activated the receptor as measured by the generation of IP₃. However, while 100 μ M 2BrP blocked the action of LA, 100 μ M 2BrP alone also resulted in an activation, indicating partial agonism (Fig. 2a). Cells transfected with the pEAK12 expression vector without the FFA₁R/GPR40 insert did not respond to LA stimulation (data not shown).

2BrP inhibits mFFA₁R/GPR40 in MIN6c4 cells

MIN6c4 is a subclone of the mouse insulinoma cell line, MIN6 (Miyazaki et al. 1990), which expresses FFA₁R/GPR40 endogenously (Kotarsky et al. 2003b, Briscoe et al. 2003, Itoh et al. 2003). This cell line has been selected because it retains the beta cell phenotype over repeated passages more consistently than the original MIN6 cells (J.M. unpublished data).

MIN6c4 cells were challenged with LA, 2BrP, and tolbutamide and assayed for receptor mediated IP₃ generation. The results were similar to those obtained in the HEK293 cells expressing the recombinant mouse receptor (Fig. 2b). LA (100 μ M) generated an IP₃ signal more than 5 times the basal level, while 2BrP and tolbutamide alone were both essentially without stimulatory effect (Fig. 2b). Unlike tolbutamide, 100 μ M 2BrP blocked the action of LA on IP₃ generation.

2BrP inhibits LA-stimulated insulin release in mouse islets

The 2BrP experiment conducted on MIN6c4 cells was repeated in mouse islets followed by measurement of insulin release. The results, which were virtually identical to those obtained for PI hydrolysis in MIN6c4 cells, demonstrated that while 100 μ M 2BrP alone had no significant effect on insulin release, it completely inhibited the release effected by LA (100 μ M) (Table 1). Tolbutamide-stimulated insulin release was unaffected by the addition of 2BrP. In order to rule out the effects of FA metabolism, a follow-up experiment using the LC-CoA synthetase inhibitor, triacsin C was performed. The LA-stimulated insulin secretion (1.91 ± 0.15 ng/islet/h) was not affected by the presence of triacsin C (1 μ M, 1.95 ± 0.16 ng/islet/h; 10 μ M 1.93 ± 0.19 ng/islet/h; n=8 in all groups, $p > 0.89$ for both concentrations of triacsin C vs LA).

Suppression of FFA₁R/GPR40 in mouse islets decreases LA-stimulated insulin release

LA-induced potentiation of insulin secretion was studied in mouse islets. In order to discriminate between insulin release mediated by FFA₁R/GPR40 and release caused by other mechanisms we designed an antisense morpholino-oligonucleotide (Heasman 2002) targeting the sequence between position -9 and -33 of the mFFA₁R/GPR40 transcript (Fig. 1).

Islets were cultured for 48 h with or without 1.4 μ M of the M40 antisense oligonucleotide. Furthermore, one group of islets was challenged for 30 min with LA (100 μ M), prior to the addition of the M40 oligonucleotide. After the culture period, the islets were washed and incubated for 60 min in 8.3 mM glucose with or without LA or 100 μ M tolbutamide, before samples were taken for measurement of the concentration of released insulin.

A significant 62% attenuation of the LA-induced stimulatory effect on insulin secretion was observed for the group pretreated with the antisense oligonucleotide alone. The combined pretreatment with LA and then M40 caused a further reduction of the LA effect on insulin secretion down to control levels. In contrast, neither GSIS nor tolbutamide-boosted GSIS was affected by the antisense oligonucleotide, even after LA pretreatment (Fig. 3). To validate that the islets were still responsive after 48 h of culturing, insulin release was measured at 1 mM glucose (0.19 ± 0.02 ng/islet/h, $n=6$) and compared to the response at 8.3 mM glucose (0.72 ± 0.04 ng/islet/h, $n=8$, $p<0.001$).

In the following experiment, the effect of increasing concentrations of LA on the insulin secretion was studied in islets treated with the mFFA₁R/GPR40 antisense nucleotide. Both untreated islets or islets treated with an unspecific morpholino-oligonucleotide were used as controls. Increasing the LA concentration from 1 μ M to 1000 μ M caused a dose-dependent increase in insulin secretion in the untreated control group. The stimulatory effect of LA on insulin secretion from islets treated with the mFFA₁R/GPR40 antisense probe was markedly reduced. No significant difference was observed between the two control groups (with or without unspecific oligonucleotide treatment) (Fig. 4). This rules out nonspecific side effects caused by the morpholino oligonucleotide chemistry.

Expression of mFFA₁R/GPR40 in pancreatic islets

In order to investigate the distribution of mFFA₁R/GPR40 within the pancreatic islet, a polyclonal antibody was raised against the C-terminal portion of mFFA₁R/GPR40. The affinity-purified antibody was first tested by FITC labeling and confocal laser microscopy on HeLa cells

stably expressing mFFA₁R/GPR40. As illustrated in Figure 5, cells expressing the receptor were stained primarily at the plasma membrane, while nontransfected reporter cells only displayed background staining.

Confocal immunofluorescence microscopy with insulin antibodies (labelled with Texas red) and mFFA₁R/GPR40 antibodies (labelled with FITC) showed that the two antigens were coexpressed in β -cells, whether viewed in intact islets (Fig. 6, a-c) or in dispersed cell groups (Fig. 6, d-f). Analysis of single cells (Fig. 6g) confirmed that mFFA₁R/GPR40 expression was primarily localized to the plasma membrane (cf. Fig. 5).

In order to verify that the observed reduction in LA-mediated insulin release was actually the consequence of a suppressed mFFA₁R/GPR40 translation, islets were subjected to LA challenge followed by antisense intervention. The islets were then visualized by confocal immunofluorescence using FFA₁R/GPR40 and insulin antibodies. The results showed that a reduction in LA-mediated insulin release was, indeed, correlated with a lower cellular expression of mFFA₁R/GPR40 (Fig. 7, a and b). The insulin staining was not affected by the antisense oligonucleotide treatment (c and d).

DISCUSSION

The mechanism generally proposed for FA-induced potentiation of insulin secretion involves metabolic coupling (Prentki et al. 1992, Corkey et al. 2000), where anaplerotic input into the tricarboxylic acid cycle generates an excess of the regulatory signalling molecule, malonyl-CoA. An increase in malonyl-CoA levels inhibits CPT-1, causing the concentration of the FA metabolite, LC-CoA, to increase. LC-CoA has been reported to affect cellular processes involved in insulin release, either directly on exocytosis (Deeney et al. 2000) or indirectly by activation of protein kinase C (Yaney et al. 2000).

Although plenty of evidence favors the “malonyl-CoA hypothesis”, arguments have also been presented against it. For example, it has been shown that neither inhibition of LC-CoA synthetase in rat islets, nor a reduction of malonyl-CoA in INS-1 cells has any effect on insulin secretion (Antinozzi et al. 1998). Intracellular application of palmitoyl-CoA has been reported unable to mimic the action of palmitate on Ca^{2+} currents and on exocytosis (Olofsson et al. 2004). Also, it has been demonstrated that FAs are capable of increasing the secretory response to non-glucose activators, such as amino acids (Dobbins et al. 1998b). When the previously unknown receptor, GPR40, was identified as a receptor for FFAs – with a high expression in β -cells and functional involvement in insulin release – it became evident that the mechanism of FA-mediated insulin release is more complex.

One of the arguments for the idea that accumulation of LC-CoA is directly involved in the potentiation mechanism of GSIS has been the finding that 2BrP stimulates insulin secretion in HIT-T15 cells (Prentki et al. 1992) and in perfused rat pancreas (Chen et al. 1994), thus

supporting the “malonyl-CoA hypothesis” by linking CPT-1 inhibition to insulin release. Originally described as an inhibitor of CPT-1 and therefore of mitochondrial long-chain FA oxidation (Chase and Tubbs 1972), 2BrP has since proven to have multiple effects on the β -cell metabolism, including inhibition of FA esterification (Parker et al. 2003). However, some authors have reported that 2BrP is unable to potentiate GSIS and also that 2BrP rather attenuates FA mediated stimulation of insulin secretion in mouse islets (Warnotte et al. 1994) and in HIT-T15 cells (Parker et al. 2003). In view of the contradictory reports regarding the effect of 2BrP on insulin secretion, it must be concluded that the mechanism of action of this substance is far from understood and, consequently, can not be used as an argument for the “malonyl-CoA hypothesis”.

Recently, it was reported that a palmitate-induced rise in cytosolic free $[Ca^{2+}]$ in mouse primary β -cells and of HIT-T15 cells was reversibly inhibited by either 100 μ M 2BrP or 100 μ M methyl palmoxirate (Remizov et al. 2003). An increase in cytosolic $[Ca^{2+}]$ is an accepted secretion signal required for palmitate-induced insulin release (Warnotte et al. 1994, Deeney et al. 2000); it is also the common signal generated by $G_{\alpha q}$ -coupled receptors like FFA₁R/GPR40. Our results agree well with the reports describing 2BrP as an inhibitor of FA-mediated insulin release and they also point out FFA₁R/GPR40 as a likely target. In the first part of the present study, we showed that 2BrP acts as an antagonist of mFFA₁R/GPR40 by blocking the LA-mediated PI hydrolysis, in experiments where receptors were expressed either transiently in HEK293 cells or endogenously in MIN6c4 cells. The discrepancy between 2BrP effects on PI hydrolysis between the cell lines can be explained by the difference in receptor densities obtained in a recombinant compared to an endogenous system. The agonist properties of partial agonists, like 2BrP, will appear more pronounced in a recombinant system with higher receptor density (Kenakin 1997). We were also

able to confirm earlier reports (Parker et al. 2003, Warnotte et al. 1994) showing that while 2BrP inhibits LA-stimulated insulin release in mouse islets, the LC-CoA synthetase inhibitor triacsin C was without effect. The fact that 2BrP not only appears as an inhibitor of FA-mediated insulin release but has now also been identified as an antagonist/partial agonist of FFA₁R/GPR40 provides a pharmacological support for our view that FFA₁R/GPR40 is an essential link in the mechanism underlying the FA-mediated potentiation of insulin secretion. However, although 2BrP acts as an antagonist on FFA₁R/GPR40, it is also a toxic substance reported to have a multitude of effects on the cell, including effects on FA metabolism and signalling. The pharmacological characterization, alone, is therefore not sufficient to link FFA₁R/GPR40 to FA-mediated potentiation of insulin secretion.

It has been argued that palmitate stimulation of GSIS proceeds by increasing [Ca²⁺] via an IP₃ independent mechanism involving enhanced Ca²⁺ influx through L-type channels (Olofsson et al. 2004). This view is not in contradiction with the concept of FFA₁R/GPR40 being essential for the mechanism of FA potentiation of insulin secretion, since the signalling pathway does not necessarily include a calcium mobilization step. It has been shown by Scholze *et al.* that G_{αi}/G_{αo}-coupled receptors are capable of stimulating pancreatic voltage gated Cav1.3 channels via a mechanism probably involving diacylglycerol acting on phorbol-activated protein kinases (Scholze et al. 2001). Another possible pathway is through G_{αq} mediated inhibition of G protein-coupled inwardly-rectifying K⁺ channels (Lei et al. 2003), which are known to be expressed in pancreatic tissue (Smith et al. 2001, Vaughn et al. 2000).

In the second part of the present report, pancreatic islets subjected to mFFA₁R/GPR40 antisense intervention displayed an attenuated LA-induced potentiation of GSIS, thus confirming earlier results on MIN6 insulinoma cells (Itoh et al. 2003). However, suppression of the receptor mRNA translation alone did not completely abolish the insulin secretory response of islets to LA. It is possible that this remaining secretory response is due to non-receptor mediated mechanisms. Another likely explanation is that, due to the slow turnover of membrane receptors, there are still enough receptors on the plasma membrane to trigger an insulin release (cf. Fig. 3). It is a well studied fact that stimulation of a GPCR generally triggers an internalisation procedure removing the receptor from the plasma membrane (Lefkowitz 2004, Gaborik and Hunyady 2004). We therefore assumed that a brief pre-stimulation with LA would enhance the effect of the antisense intervention. Accordingly, the remaining part of the LA-induced stimulatory effect completely disappeared when the islets were challenged for 30 min with LA prior to the 48-hour antisense exposure period. The fact that a tolbutamide-induced insulin release (Proks et al. 2002) was unaffected by both antisense oligonucleotide and LA indicates that the suppressive effect was specific.

In the final part of our study the distribution of mFFA₁R/GPR40 within the islet was established using confocal immunofluorescence microscopy. It was found that the receptor distribution to a great extent overlapped with the distribution of insulin immunoreactivity. This agrees with the observations of both Briscoe *et al.* (Briscoe et al. 2003) and Itoh *et al.* (Itoh et al. 2003). An important observation in our antisense interventions was that a decrease in islet mFFA₁R/GPR40 immunostaining strongly correlated with the reduction in LA-stimulated insulin secretion.

An argument that has been raised against a functional relevance of FFA₁R/GPR40 (Poitout 2003) has been that receptor activation in the presence of albumin has not yet been demonstrated. It should be noted that all our experiments on mouse islets included 1% FA-free BSA in the culture as well as incubation media. In addition, a recent study of FFA₁R/GPR40, in human breast cancer cells demonstrated that receptor mediated calcium activation occurs in the presence of 0.5% albumin (Yonezawa et al. 2004).

Long-term elevation FA levels has a deleterious effects on β -cell function, often referred to as lipotoxicity (Robertson et al. 2004). An important question is if FFA₁R/GPR40 is involved also in the long-term lipotoxic effect of FA. In the lipotoxicity research there is an increasing interest in the control of the β -cell lipid metabolism by lipid-sensing transcription factors like PPAR α , PPAR δ , PPAR γ and SREBP-1c (Fatehi-Hassanabad and Chan 2005). An important future task will be to show how the membrane bound lipid receptor FFA₁R/GPR40 interrelate with the nuclear PPAR and SREBP-1c receptors.

Taken together, our results show that activation of FFA₁R/GPR40 constitutes an important, if not essential, part of fatty acid mediated stimulation of insulin secretion. The emerging picture of the mechanism indicates a complex process involving both metabolic coupling and receptor-mediated events, where we believe that the latter is of the utmost importance. It is obvious that more research is needed to determine exactly how these components relate to each other.

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TABLES

Table 1 Insulin secretion from isolated mouse pancreatic islets incubated in 8.3 mM glucose

Treatment ^a	Insulin (ng/islet/h) ^b
control	0,48 ± 0,04 ^c
LA	1,46 ± 0,09
LA/2BrP	0,64 ± 0,07 ^c
2BrP	0,60 ± 0,08
tolb	1,07 ± 0,14 ^d
tolb/2BrP	1,24 ± 0,13

^a All substances were used at 100 µM

^b Mean ± SEM from 4 experiments (n=8)

^c p<0.001 vs LA

^d p<0.01 vs control

FIGURES AND LEGENDS

Fig. 1

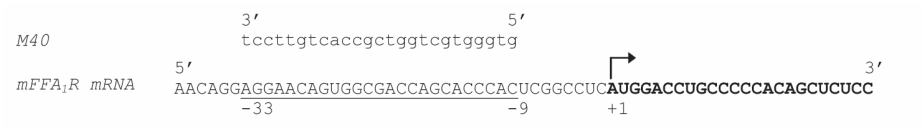


Fig. 1 Schematic representation of mFFA₁R/GPR40 mRNA in the region of the start of the open reading frame (+1). The M40 morpholino oligonucleotide (lower case) hybridizes with the mRNA between positions -33 and -9 (underlined) relative to the mFFA₁R/GPR40 coding sequence (bold).

Fig. 2

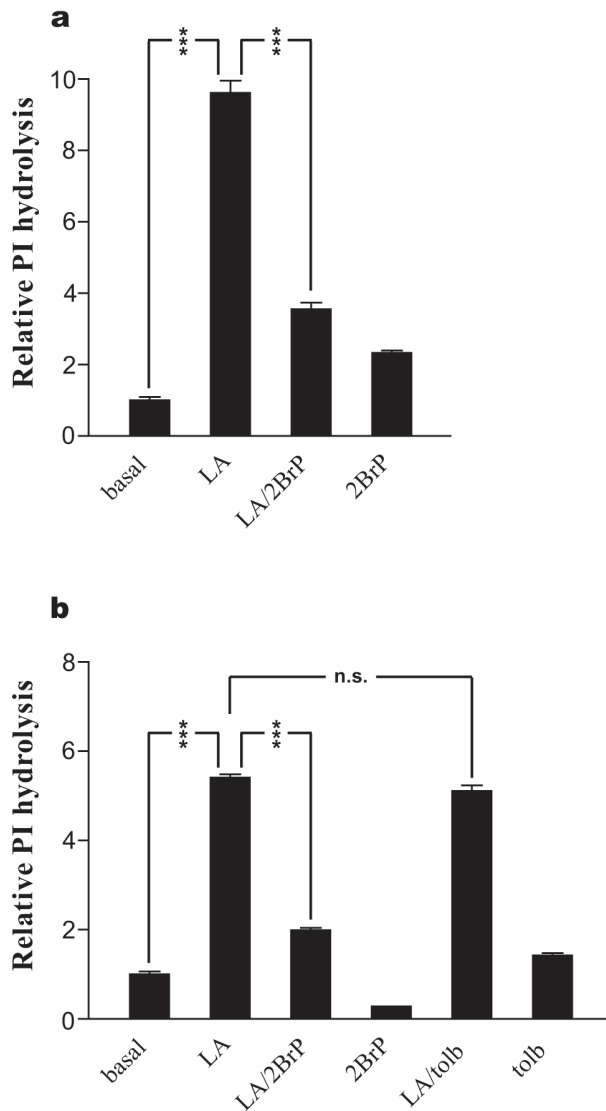


Fig. 2 PI hydrolysis in response to LA and 2BrP. **a** In transiently transfected HEK293 cells, LA increased the basal level of PI hydrolysis ten-fold. This increase was counteracted by 2BrP, which by itself caused a two-fold increase, indicating a partial agonism. **b** In MIN6c4 cells, stimulation with LA caused an almost six-fold increase in PI hydrolysis compared to basal levels. This effect was abolished by simultaneous addition of 2BrP. 2BrP alone lowered basal IP levels in MIN6c4 cells. Tolbutamide (tolb) had no effect, neither on LA-induced nor on basal levels of IP production. All substances in these experiments were used at 100 μ M. Data are means + SEM from representative experiments repeated on 2 occasions (n=3). *** = $p < 0.001$, n.s = not significant

Fig. 3

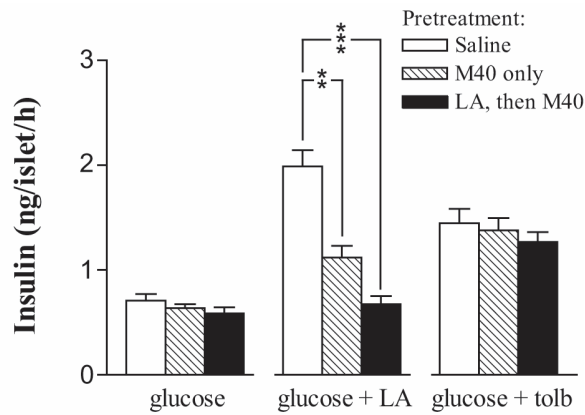


Fig. 3 Islets subjected to antisense treatment with the mFFA₁R/GPR40 specific morpholino, M40 exhibit a blunted response to LA. Figure showing insulin release from islets in response to different stimulation; glucose (8.3 mM), glucose + LA (100 μ M), and glucose + tolbutamide (100 μ M). Prior to this stimulation, the islets received either a saline pretreatment (white bars), a 48 h pretreatment with the M40 antisense oligonucleotide (hashed bars), or a combination of a 30 min pretreatment with 100 μ M LA followed by a 48 h incubation with the morpholino, M40 (black bars). Insulin release in response to glucose stimulation alone remained unaffected by the changes in pretreatment, whereas the M40 pretreatment significantly reduced the LA-mediated augmentation of GSIS. The combined LA + M40 pretreatment reduced the augmentation even further. The effect of tolbutamide is unaltered by the presence of the M40 oligonucleotide. Means + SEM for 8 batches of islets per datapoint. Each batch contained 8-12 islets, incubated for 60 min in the presence of 8.3 mM glucose +/- LA or tolbutamide. *** = $p < 0.001$ and ** = $p < 0.01$

Fig. 4

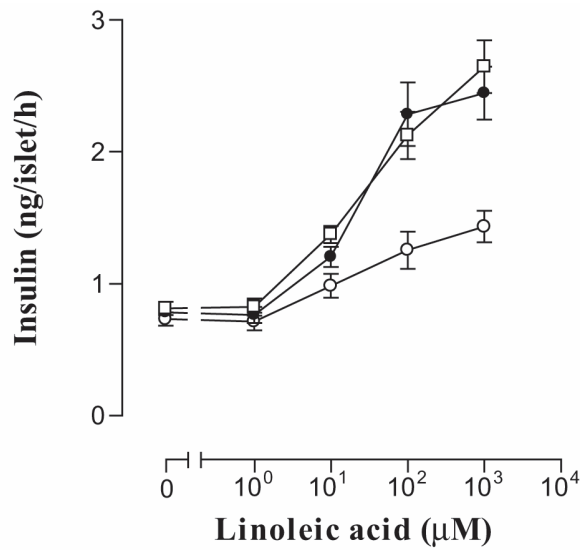


Fig. 4 Treatment of islets with the M40 morpholino caused a marked reduction in LA-stimulated insulin secretion (white circles). The control morpholino failed to interfere with insulin secretion (black circles) compared to untreated islets (white boxes). Islets (12 in each vial) were incubated for 60 min in the presence of 8.3 mM glucose alone or 8.3 mM glucose and an increasing concentration of LA, as indicated in the figure. Values are means + SEM for 6-8 different experiments performed at different occasions.

Fig. 5

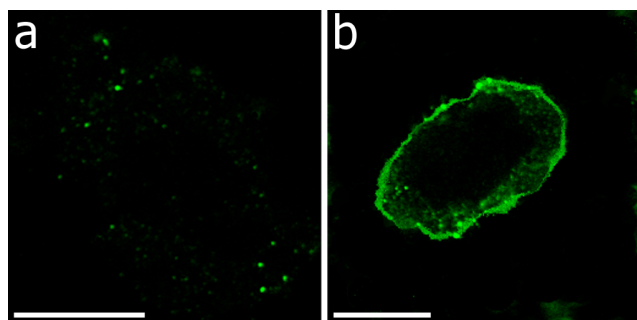


Fig. 5 Specificity of the mFFA₁R/GPR40 antibody. Confocal microscopy image of an untransfected HeLa cell (left) and an HeLa cell stably expressing mFFA₁R/GPR40 cDNA (right). Scale bars are 10 μM.

Fig. 6

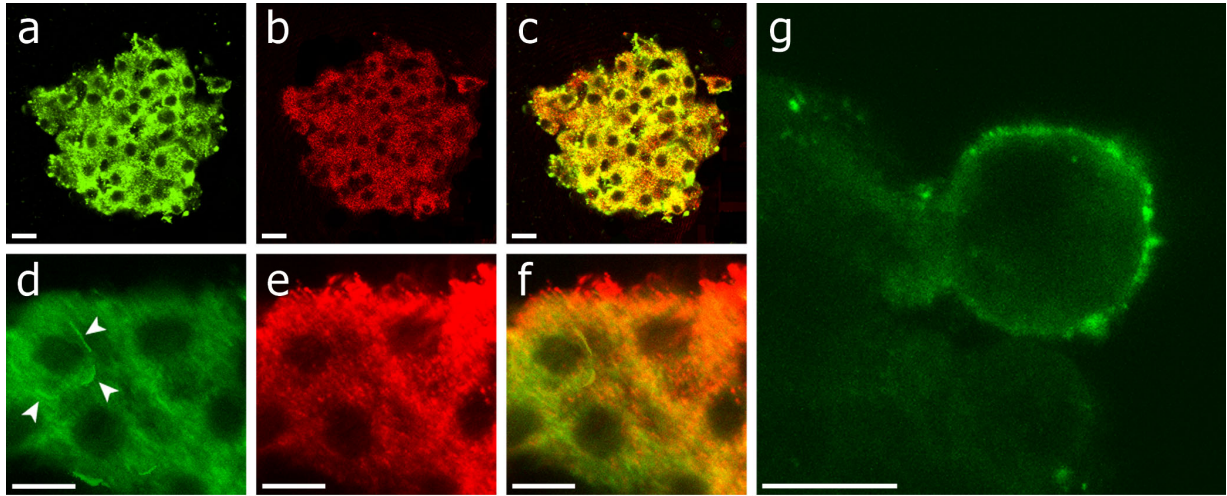


Fig. 6 Confocal microscopy images of an intact islet (a-c) or dispersed islet cells (d-f) demonstrating the expression of mFFA₁R/GPR40 (a and d), insulin (b and e), and co-expression of both (c and f). An increased magnification shows that mFFA₁R/GPR40 is expressed on the β -cell surface (g). Arrowheads (d) illustrate sites of FFA₁R/GPR40 expression in the membrane of one β -cell. Scale bars are 10 μ M.

Fig. 7

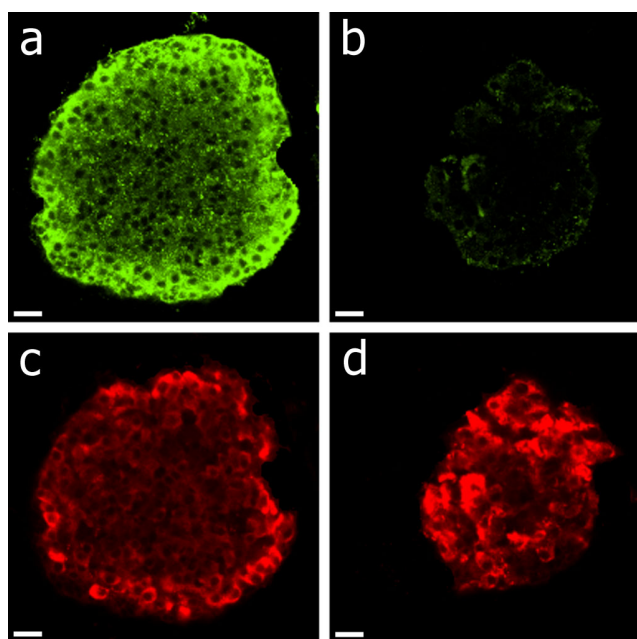


Fig. 7 Confocal microscopy images of mouse islets. The top panels show islets stained for mFFA₁R/GPR40 expression, both untreated (a) and treated for 30 min with 100 μ M LA and then for 48 hours with the morpholino, M40 (b). The bottom panels show the same islets, either untreated (c) or subjected to identical LA/M40 treatment (d), stained for insulin. Scale bars are 10 μ M.