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Uncovering the Pharmacology of the G Protein-Coupled Receptor GPR40: High Apparent Constitutive Activity in Guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate Binding Studies Reflects Binding of an Endogenous Agonist

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ABSTRACT

In cells lacking expression of Ca^{2+}-mobilizing G proteins, co-expression of human GPR40 and $G\alpha_q$ allowed medium- and long-chain fatty acids to elevate intracellular [Ca^{2+}]. This was also observed when human embryonic kidney (HEK) 293 cells were transfected with a GPR40-$G\alpha_q$ fusion protein. The kinetic of elevation of intracellular [Ca^{2+}] slowed with increasing fatty acid chain length, suggesting different ligand on-rates, whereas the addition of fatty acid-free bovine serum albumin reduced signals, presumably by binding the fatty acids. To allow effective ligand equilibration, GPR40-$G\alpha_q$ was used in guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) binding assays. After expression of GPR40-$G\alpha_q$ in HEK293 cells and membrane preparation basal binding of [^{35}S]GTPγS in $G\alpha_q$ immunoprecipitates was high and not elevated substantially by fatty acids. However, treatment of membranes with fatty acid-free bovine serum albumin reduced the basal [^{35}S]GTPγS binding in a concentration-dependent manner and allowed the responsiveness and pharmacology at GPR40 of each of the fatty acids thiazolidinediones and a novel small-molecule agonist to be uncovered. Membranes of rat INS-1E cells that express GPR40 endogenously provided similar observations. The high apparent constitutive activity of GPR40-$G\alpha_q$ was also reversed by a small-molecule GPR40 antagonist, and basal [^{35}S]GTPγS binding was prevented by the selective $G\alpha_q$/$G\alpha_{11}$ inhibitor YM-254890. The current studies provide novel insights into the pharmacology of GPR40 and indicate that G protein-coupled receptors which respond to fatty acids, and potentially to other lipid ligands, can be occupied by endogenous agonists before assay and that this may mask the pharmacology of the receptor and may be mistaken for high levels of constitutive activity.

GPR40 is a member of a small group of G protein-coupled receptors (GPCRs) clustered at chromosome 19q13.1 in humans (Sawzdargo et al., 1997) that generate signals in response to free fatty acids of various chain lengths (Brown et al., 2005; Milligan et al., 2006). GPR40 has attracted considerable attention (Gromada, 2006; Milligan et al., 2006), because expression profiling has demonstrated its mRNA to be expressed at high levels in the insulin-secreting β cells of the pancreas and in a range of pancreatic β-cell lines (Briscoe et al., 2003; Itoh et al., 2003; Itoh and Hinuma, 2006) and in human tissue (Tomita et al., 2006). This has suggested a potential role for GPR40 in the regulation of β-cell responsiveness to free fatty acids and hence as a potential therapeutic target in the regulation of insulin secretion and diabetes. In support of this, knockdown of GPR40 levels in the transformed murine β-cell line MIN6 via small interfering RNAs or antisense treatment greatly reduces free fatty acid-mediated insulin release (Itoh et al., 2003; Salehi et al., 2005; Shapiro et al., 2005).

Such observations have focused attention on the pharmacology of GPR40 and initiated efforts to identify small-molecule agonists and antagonists of this receptor (Briscoe et al., 2006; Garrido et al., 2006). Recent reports have described both agonists and antagonists, with the agonists being able to enhance glucose-stimulated insulin secretion in MIN6

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ABBREVIATIONS: GPCR, G protein-coupled receptor; BSA, bovine serum albumin; CFP, cyan fluorescent protein; [^{35}S]GTPγS, guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate; GW1100, (ethyl 4-[5-{[2-(ethyloxy)-5-pyrimidinyl]methyl}-2-{[4-fluorophenyl) methyl]thio}-4-oxo1(4*H*)-pyrimidinyl]-benzoate; PPAR, peroxisome proliferator-activated receptor; YFP, yellow fluorescent protein; CQ, antiserum directed against the C-terminal decapeptide of G_q/G_{11}; HEK, human embryonic kidney; PCR, polymerase chain reaction.

cells (Briscoe et al., 2006). It is interesting that, although well-appreciated to be an agonist of the PPARγ isoforms of the peroxisome proliferator-activated receptor group of nuclear hormone receptors (Staels and Fruchart, 2005; Kepez et al., 2006; Semple et al., 2006), the insulin-sensitizing thiazolidinedione, rosiglitazone, also has been shown recently to be an agonist at rat GPR40 (Kotarsky et al., 2003a,b).

The majority of studies on the pharmacology of GPR40 have taken advantage of the selective coupling of this receptor to G_q family G proteins and hence to the elevation of intracellular [Ca^{2+}] (Briscoe et al., 2003; Itoh et al., 2003; Briscoe et al., 2006). However, because many of the free fatty acids that display agonism at GPR40 show relatively low potency, such approaches are less than ideal because of well-appreciated issues of slow ligand on-rates and therefore the potential effects of hemiequilibrium in competition studies (Christopoulos et al., 1999). A number of GPCRs also display a substantial degree of agonist-independent constitutive activity (Milligan, 2004; Bond and IJzerman, 2006), and pharmacological studies based on the dynamics of cellular Ca^{2+} mobilization are also well known to be inappropriate to detect such activity. Although historically, the use of [^{35}S]GTPγS binding studies was limited to GPCRs that couple predominantly to the G_i family of G proteins (Harrison and Traynor, 2003), combinations of the use of end of assay immunocapture studies and the construction of GPCR-G protein fusions have revolutionized this approach (Milligan, 2003; Milligan et al., 2004).

How much of measured "constitutive" activity of GPCRs might reflect the unappreciated and undetected presence of endogenous agonists has been a question that has interested, and indeed perplexed, pharmacologists since the earliest observations of such activity and the definition of "inverse" agonists as agents able to suppress inherent constitutive activity (Milligan et al., 1995). By using combinations of Ca^{2+} mobilization and [^{35}S]GTPγS binding techniques and cell systems that can express GPR40 in an entirely inducible fashion, we now explore details of the pharmacology of human GPR40. We demonstrate that the measured high constitutive activity of GPR40 in [^{35}S]GTPγS binding studies is an artifact that reflects the presence of an endogenous agonist that can be removed by treatment with fatty acid-free serum albumin to uncover a robust pharmacology. We show this to be the case in the transformed pancreatic β-cell line INS-1E, which expresses GPR40 endogenously, and in transfected cell systems. Furthermore, the related receptor GPR41 that responds only to less hydrophobic, short-chain fatty acids (Brown et al., 2003) does not display high levels of apparent constitutive activity that can be eliminated by treatment with similar concentrations of fatty acid-free serum albumin.

Materials and Methods

Materials

[^{35}S]GTPγS was from PerkinElmer (Boston, MA). Materials for tissue culture were from Sigma-Aldrich (Gillingham, Dorset, UK) and Invitrogen (Paisley, UK). Fatty acid-free BSA was from Roche Applied Science (Lewes, East Sussex, UK). Fatty acids were from Sigma and were dissolved in ethanol. Thiazolidinediones were from Axxora (Bingham, Nottingham, UK), and YM-254890 (Takaski et al., 2004) was the kind gift of Astellas Pharma Inc. (Osaka, Japan).

Generation of GPR40 Constructs

GPR40-FLAG. Primers to remove the stop codon of human GPR40 and to introduce the FLAG epitope sequence were used to generate C-terminally tagged GPR40-FLAG: sense, 5′-CCGGGTACCATGGACCTGCCCCCGCAGCTCTCCTTC-3′; antisense, 5′-CTAGTCTAGACTA***AGACTTATCATCGTCGTCCTTGTAGTC***CTTCTGGGACTTGCCCCCTTGCGT-3′. The region encoding the FLAG epitope tag is shown in boldface italic type. The KpnI and XbaI sites present in the sense and antisense primers, respectively, are underlined, and the amplified fragment was digested and ligated into pcDNA3.

GPR40-c-myc. Primers to remove the stop codon of GPR40 and to introduce the c-myc epitope sequence were used to generate C-terminally tagged GPR40-c-myc: sense, 5′-CCGGGTACCATGGACCTGCCCCCGCAGCTCTCCTTC-3′; antisense, 5′-CTAGTCTAGACTA***CAGATCTTCTTCAGAAATAAGTTTTTGTTC***CTTCTGGGACTTGCCCCCTTGCGT-3′. The region encoding the c-myc epitope tag is shown in boldface italic type. The KpnI and XbaI sites present in the sense and antisense primers, respectively, are underlined, and the amplified fragment was digested and ligated into pcDNA3.

GPR40-CFP. Primers were designed to amplify GPR40 and to remove the stop codon: sense, 5′-CCCCCAAGCTTCCACCATGGACCTGCCCCCGCAGCTC-3′; antisense, 5′-AAAAGATATCCTTCTGGGACTTGCCCCCTTGC-3′. The HindIII and EcoRV sites present in the sense and antisense primers, respectively, are underlined. The amplified fragment was digested and ligated into pcDNA3 in frame with eCFP ligated between NotI and XhoI.

GPR40-YFP. Primers were designed to amplify GPR40 and to remove the stop codon: sense, 5′-CCCCCAAGCTTCCACCATGGACCTGCCCCCGCAGCTC-3′; antisense, 5′-AAAAGATATCCTTCTGGGACTTGCCCCCTTGC-3′. The HindIII and EcoRV sites present in the sense and antisense primers, respectively, are underlined. The amplified fragment was digested and ligated into pcDNA3, in frame, with eYFP ligated between NotI and XhoI.

GPR40-Gα_q. Primers were designed to amplify GPR40 and to remove the stop codon: sense, 5′-CCCCCAAGCTTCCACCATGGACCTGCCCCCGCAGCTC-3′; antisense, 5′-AAAAGATATCCTTCTGGGACTTGCCCCCTTGC-3′. The HindIII and EcoRV sites present in the sense and antisense primers, respectively, are underlined. Primers to amplify Gα_q were also designed: sense, 5′-AAAAGATATCACTCTGGAGTCCATCATGGCGTGC-3′; antisense, 5′-TTTTTTCCTTGCGGCCGCTTAGACCAGATTGTACTCCTTCAG-3′. The EcoRV and NotI sites present in the sense and antisense primers, respectively, are underlined. The amplified fragments of GPR40 and Gα_q were digested with the appropriate enzymes and ligated, in frame, into pcDNA3.

Generation of Stable Flp-In T-REx HEK293 Cells

Flp-In T-REx HEK293 cells (Milasta et al., 2006; Canals et al., 2006) were maintained in Dulbecco's modified Eagle's medium without sodium pyruvate, 4500 mg/l glucose and L-glutamine, supplemented with 10% (v/v) fetal calf serum, 1% antibiotic mixture, and 10 μg/ml blasticidin at 37°C in a humidified atmosphere of air/CO_2 (19:1). To generate Flp-In T-REx HEK293 cells able to inducibly express human GPR40-YFP, the cells were transfected with a mixture containing this cDNA in pcDNA5/FRT/TO vector and the pOG44 vector (1:9) using LipofectAMINE (Invitrogen) according to the manufacturers' instructions. After 48 h, the medium was changed to medium supplemented with 200 μg/ml hygromycin B to initiate the selection of stably transfected cells. After isolation of individual clones, cells were treated with 1 μg/ml doxycycline for 24 h to induce expression of GPR40-YFP from the Flp-In locus.

INS-IE Cells. The transformed rat pancreatic β-cell line INS-1E was maintained in RPMI 1640 medium with L-glutamine supplemented with 10% fetal calf serum, 10 mM HEPES, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, and 1% antibiotic mixture at 37°C in a humidified atmosphere of air/CO_2 (19:1).

Cell Membrane Preparation

Harvested pellets from transfected HEK293 cells, Flp-In T-REx HEK293 cells, and INS-IE cells maintained at −80°C were thawed and resuspended in 10 mM Tris and 0.1 mM EDTA, pH 7.4 (Tris/EDTA buffer). The cells were homogenized by 50 passes of a glass-on-Teflon homogenizer. The resulting suspension was centrifuged at 1200*g* for 10 min to remove unbroken cells and nuclei. The supernatant was subsequently centrifuged at 218,000*g* for 30 min in a Beckman Optima TLX Ultracentrifuge (Palo Alto, CA). The resulting pellets were resuspended in Tris/EDTA buffer and passed 10 times through a 25-gauge needle. Protein concentration was determined, and the membranes were diluted to 1 μg/μl and stored at −80°C until required.

[^{35}S]GTPγS Binding Assays

[^{35}S]GTPγS binding experiments were initiated by the addition of 2.5 μg of cell membranes to an assay buffer (20 mM HEPES, pH 7.4, 3 mM $MgCl_2$, 100 mM NaCl, 1 μM GDP, 0.2 mM ascorbic acid, and 50 nCi [^{35}S]GTPγS) containing the given concentration of agonist or antagonist. Nonspecific binding was determined in the above condition with the addition of 100 μM GTPγS. Reactions were incubated for 30 min at 30°C and were terminated by the addition of 500 μl of ice-cold buffer containing 20 mM HEPES, pH 7.4, 3 mM $MgCl_2$, 100 mM NaCl, and 0.2 mM ascorbic acid. The samples were centrifuged at 16,000*g* for 10 min at 4°C. The resulting pellets were resuspended in solubilization buffer (100 mM Tris, 200 mM NaCl, 1 mM EDTA, and 1.25% Nonidet P-40) plus 0.2% SDS. Samples were precleared with Pansorbin followed by immunoprecipitation with the C-terminal $G\alpha_q/G\alpha_{11}$ antiserum CQ (Mitchell et al., 1991). Finally, the immunocomplexes were washed once with solubilization buffer, and bound [^{35}S]GTPγS was estimated by liquid scintillation spectrometry. In studies using GPR41-$G\alpha_{i3}$, this fusion protein was recovered at the end of assay by immunoprecipitation with a $G\alpha_{i3}$-selective antiserum (McClue et al., 1992).

Single Cell Calcium Assays

HEK293 cells and EF88 cells (mouse embryo fibroblasts derived from $G\alpha_q$ + $G\alpha_{11}$ knockout mouse lines) (Liu et al., 2002) grown on poly(D-lysine)-coated coverslips were transiently transfected to express the construct of interest. For Flp-In T-REx HEK293 cells, these were treated with or without 1 μg/ml doxycycline. Twenty-four hours after transfection or cell treatment, cells were loaded with the calcium sensitive dye Fura-2, 1.5 μM Fura-2 was added to normal growth media and the cells incubated at 37°C for 30 min. After loading of the dye, coverslips were placed into a microscope chamber containing physiological saline solution and illuminated with an ultrahigh point intensity 75-W xenon arc lamp (Optosource, Cairn Research, Faversham, Kent, UK) and imaged using a Nikon Diaphot inverted microscope equipped with a Nikon (Tokyo, Japan) 40× oil immersion Fluor objective lens (numerical aperture = 1.3) and a monochromator (Optoscan, Cairn Research), which was used to alternate the excitation wavelength between 340/380 nm and to control the excitation band pass (340 nm band pass = 10 nm; 380 nm band pass = 8 nm). Fura-2 fluorescence emission at 510 nm was monitored using a high-resolution interline-transfer cooled digital charge-coupled device camera (Cool Snap-HQ, Roper Scientific/Photometrics, Tucson, AZ). MetaFluor imaging software (Universal Imaging Corp., Downing, PA) was used for control of the monochromator, charge-coupled device camera, and for processing of the cell image data. Sequential images (2 × 2 binning) were collected every 2 s, and exposure to excitation light was 100 ms/image. Agonist was added after 60 s (after 30 images) for 60 s using a perfusion system. MetaFluor software was used to analyze the images.

Calcium Assays Using Cell Populations

Calcium assays were performed using Flp-In T-REx HEK293 cells harboring GPR40-YFP that were treated with or without 1 μg/ml doxycycline. Cells were grown in wells of a 96-well microtiter plate. Twenty-four hours after transfection, cells were loaded with the calcium-sensitive dye Fura-2 as above, and effect of potential ligands assessed using a FLEXStation (Molecular Devices, Sunnyvale, CA).

Reverse Transcriptase-PCR to Detect Rat GPR40 mRNA

Total RNA was extracted from INS-1E cells using the RNeasy MiniPrep procedure (QIAGEN Inc., Crawley, UK). First-strand cDNA was synthesized using the first strand cDNA Synthesis kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Detection of specific mRNA transcripts was carried out by PCR using 50 ng of cDNA and 200 nM oligonucleotides corresponding to rat GPR40 or rat cyclophilin. Primers used for PCR are as follows: rat GPR40, 5′-CCCTGCCCGACTCAGTTTC-3′ and 5′-GGCAGCCCACATAGCAGAA-3′; rat cyclophilin, 5′-TCACCATCTCCGACTGTGGA-3′ and 5′-AAATGCCCGCAAGTCAAAGA-3′. Cycling conditions were as follows: 10 min at 95°C and 40 cycles of 1 min at 95°C, 1 min at 52°C, 2 min at 72°C, followed by 5 min at 72°C.

Results

When coexpressed with $G\alpha_q$ in mouse embryo fibroblasts, previously called EF88 cells (Liu et al., 2002), which lack endogenous expression of both of the Ca^{2+}-mobilizing G proteins $G\alpha_q$ and $G\alpha_{11}$, the human GPCR GPR40 allowed rapid and transient elevation of intracellular [Ca^{2+}] ([Ca^{2+}]$_i$) in response to the addition of a range of medium- and longer-chain fatty acids, including caproic (C6:0) acid (Fig. 1a).

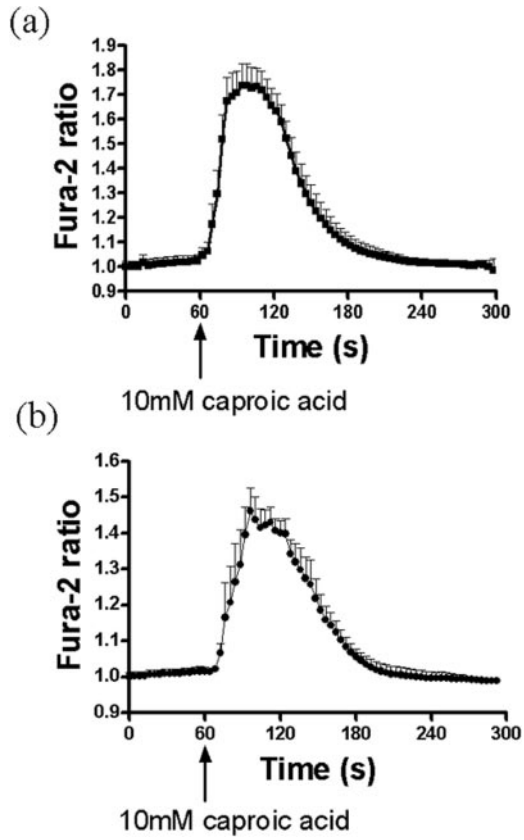

Fig. 1. GPR40 activates $G\alpha_q$ and causes elevation in intracellular [Ca^{2+}]. EF88 mouse embryo fibroblasts lacking expressing of both $G\alpha_q$ and $G\alpha_{11}$ (Liu et al., 2002) were transiently transfected to express both human GPR40 and $G\alpha_q$ (a) or a GPR40-$G\alpha_q$ fusion protein (b). After loading with Fura-2, caproic acid (10 mM) was added at the 60-s time point and the alteration in Fura-2 fluorescence monitored over the following 240 s as described under *Materials and Methods*. Data represent means ± S.E.M. from 20 individual cells.

Similar results were obtained when GPR40 and $G\alpha_q$ were expressed in these cells as a single open-reading frame fusion protein, in which the N terminus of $G\alpha_q$ was linked to the intracellular C-terminal tail of GPR40 (Fig. 1b). These experiments confirmed, as for many other GPCRs (Milligan et al., 2004), that C-terminal fusion of a cognate G protein allows the expression of an active bifunctional polypeptide.

Cotransfection of $G\alpha_q$ was not required for caproic acid to generate a Ca^{2+} signal when GPR40 was expressed in HEK293 cells (Fig. 2a) because these express both $G\alpha_q$ and $G\alpha_{11}$ endogenously. A wide range of modifications at the C-terminal tail of GPR40, including the introduction of c-myc or FLAG epitope tags and the in-frame fusion of either cyan (CFP) or yellow (YFP) fluorescent proteins could also be tolerated without loss of agonist function (Fig. 2a). Caproic acid has been described previously as a low-affinity agonist for GPR40 and the shortest-chain length fatty acid able to activate this receptor (Briscoe et al., 2003). A range of other fatty acids was tested for their capacity to elevate $[Ca^{2+}]_i$ in HEK293 cells transiently transfected to express the GPR40-CFP fusion protein. Each of lauric (C12:0) acid, palmitic (C16:0) acid, and elaidic (C18:2) acid elevated $[Ca^{2+}]_i$ (Fig. 2b) only in positively transfected cells displaying CFP fluorescence (see below), but the kinetic of signal generation in response to the individual fatty acids was very distinct (Fig. 2b).

Slower elevation of $[Ca^{2+}]_i$ was observed with longer-chain length, with the delayed response to elaidic acid being particularly obvious. Although not examined in detail, this may reflect differences in affinity/ligand association rate or variations in solubility characteristics. Because the bulk of fatty acids in blood are complexed with albumin and other serum proteins, then, as anticipated, the addition of increasing amounts of fatty acid-free BSA along with lauric acid (Fig. 2c) or other free fatty acids (data not shown) reduced the extent of $[Ca^{2+}]_i$ release in a concentration-dependent manner, such that with 0.5% (w/v) BSA, no detectable signal was produced by 100 μM lauric acid (Fig. 2c).

Medium- and long-chain-free fatty acids are the accepted endogenous agonists of GPR40 (Briscoe et al., 2003; Itoh et al., 2003; Kotarsky et al., 2003). However, as described previously for rat GPR40 (Kotarsky et al., 2003a,b), thiazolidinedione ligands such as rosiglitazone and troglitazone that are usually described as agonists of the PPARγ group of peroxisome proliferator-activated receptors also activated the human GPR40-YFP fusion, causing rapid and transient

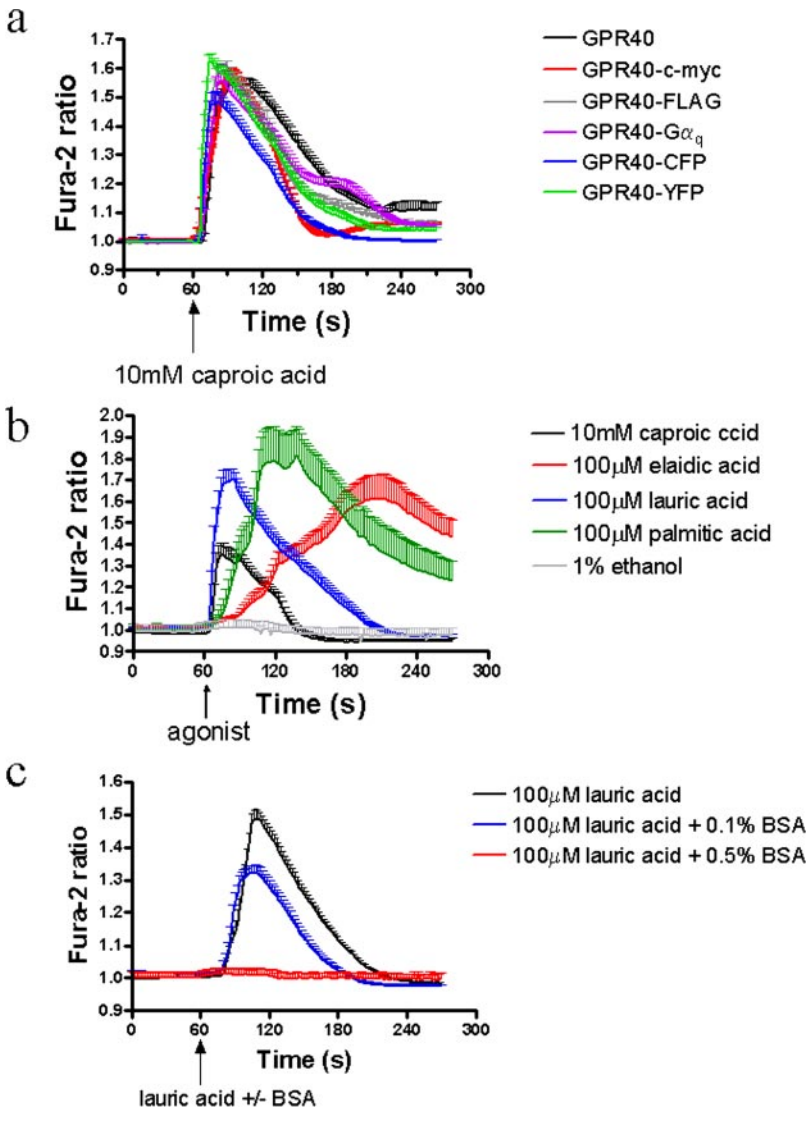

Fig. 2. Modifications to the C-terminal tail of GPR40 do not limit function, but responses to different free fatty acids display distinct kinetics. a, HEK293 cells were transfected to transiently express GPR40 (black), GPR40-c-myc (red), GPR40-FLAG (gray), GPR40-$G\alpha_q$ (purple), GPR40-CFP (blue), or GPR40-YFP (green). After loading with Fura-2, caproic acid (10 mM) was added at the 60-s time point, and the alteration in Fura-2 fluorescence monitored over time as in Fig. 1 and *Materials and Methods*. b, HEK293 cells transiently expressing GPR40-CFP as in *a* were exposed to 100 μM lauric acid (blue), palmitic acid (green), elaidic acid (red), 10 mM caproic acid (black), or 1% ethanol (gray) at the 60-s time point, and the alteration in Fura-2 fluorescence was monitored over time. c, cells as in *b* were treated with 100 μM lauric acid in the absence (black) or presence of 0.1% (blue) or 0.5% (red) fatty acid-free BSA. Data represent the means ± S.E.M. from 20 individual cells.

elevation of $[Ca^{2+}]_i$ only in HEK293 cells positively transfected with GPR40-YFP (Fig. 3, a and b). To confirm that expression of GPR40 was required to allow the elevation of $[Ca^{2+}]_i$ in response to thiazolidinediones, we generated stable clones of Flp-In T-REx-HEK293 cells (Canals et al., 2006; Milasta et al., 2006) in which GPR40-YFP was cloned into the Flp-In locus. These cells allow expression from the Flp-In locus only upon the addition of tetracycline or the related antibiotic doxycycline. In the absence of antibiotic treatment, no autofluorescence corresponding to YFP could be detected, and the addition of troglitazone did not result in elevation of $[Ca^{2+}]_i$ (Fig. 3c). By contrast, after treatment with doxycycline (1 μg/ml for 24 h), GPR40-YFP was present at the surface of the cells, and troglitazone produced a robust and rapid elevation of $[Ca^{2+}]_i$ (Fig. 3c).

Single-cell $[Ca^{2+}]$-imaging studies are not well-suited for examining ligand concentration-dependence, and low-affinity ligands will not achieve binding equilibrium over the time course of such studies. We therefore used the GPR40-$G\alpha_q$ fusion protein in $[^{35}S]GTP\gamma S$ binding studies. We have shown previously that, in combination with an end of assay immunocapture or enrichment step, activation of $G\alpha_q$-coupled GPCRs can easily be monitored using such fusion proteins (Carrillo et al., 2002; Milligan et al., 2004). After expression of GPR40-$G\alpha_q$ in HEK293 cells and membrane preparation, $[^{35}S]GTP\gamma S$ binding studies were performed with subsequent immunoprecipitation using the anti-$G\alpha_q$/$G\alpha_{11}$ antiserum CQ (Mitchell et al., 1991). Unlike a range of other GPCR-$G\alpha_q$ or GPCR-$G\alpha_{11}$ fusion proteins we have generated and studied, in which the loading of $[^{35}S]GTP\gamma S$ is very low in the absence of receptor stimulation (Carrillo et al., 2002; Milligan et al., 2004), high levels of $[^{35}S]GTP\gamma S$ were present in the GPR40-$G\alpha_q$ immunoprecipitates in the absence of an added agonist (Fig. 4a). Furthermore, the presence of either palmitic acid or elaidic acid (30 μM) did not increase this (Fig. 4a). This high level of basal $[^{35}S]GTP\gamma S$ binding was a direct reflection of guanine nucleotide exchange on the G protein because the presence of $[^{35}S]GTP\gamma S$ in these immunoprecipitates was virtually eliminated by the presence of the selective $G\alpha_q$ and $G\alpha_{11}$ inhibitor YM-254890

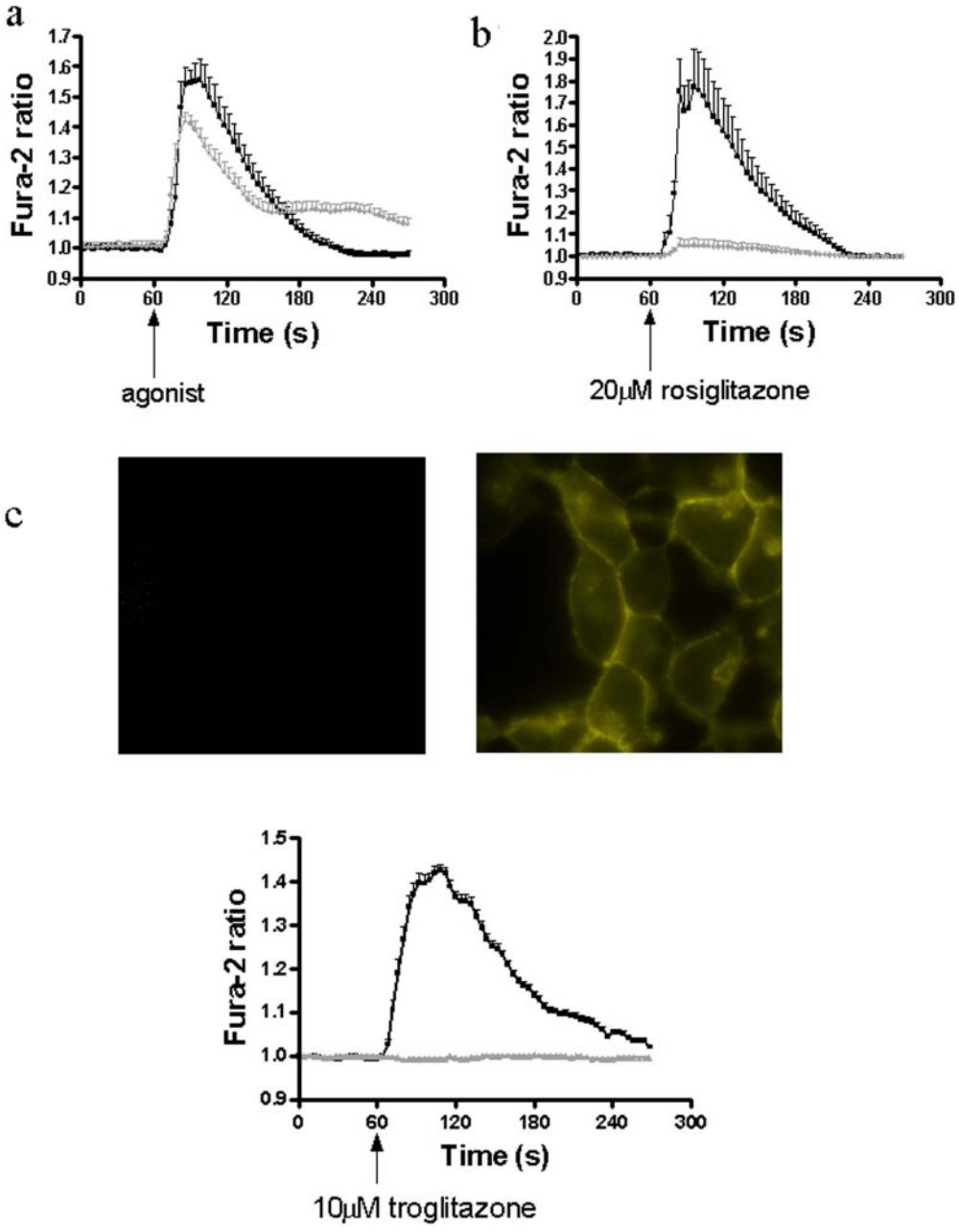

Fig. 3. The thiazolidinediones rosiglitazone and troglitazone are agonists at human GPR40. a, HEK293 cells transiently expressing GPR40-YFP were exposed to the thiazolidinediones rosiglitazone (black) and troglitazone (gray) (20 μM), and elevation of intracellular $[Ca^{2+}]$ was monitored over time as in Fig. 1. b, HEK293 cells were transiently transfected with GPR40-YFP. As monitored by YFP fluorescence, individual cells were positively transfected (black) or not (gray). Rosiglitazone (20 μM) was added and only caused a significant elevation of $[Ca^{2+}]$ in cells expressing GPR40-YFP. c, cells of a clone of Flp-In T-REx-HEK293 cells with GPR40-YFP located at the Flp-In locus were grown on glass coverslips and treated with (right) or without (left) 1 μg/ml doxycycline for 24 h. Cells were visualized to detect expression of GPR40-YFP (top) and alterations in $[Ca^{2+}]_i$ to 10 μM troglitazone monitored (bottom) in +DOX (black) and −DOX (gray) cells.

(Fig. 4b), which blocks guanine nucleotide exchange on these G protein α subunits (Takasaki et al., 2004; Canals et al., 2006).

These observations were potentially consistent with GPR40 displaying high levels of constitutive, agonist-independent activity. Addition of the small-molecule GPR40 antagonist GW1100 (Briscoe et al., 2006) (also designated as SB376752) (10 μM) also markedly inhibited the basal loading of [^{35}S]GTPγS onto GPR40-Gα_q (Fig. 4b). This observation is also potentially consistent with GPR40 displaying high levels of constitutive activity if, as with many small-molecule blockers of GPCR function, GW1100 acts as a GPR40 inverse agonist rather than a neutral antagonist. However, given that free fatty acids are endogenous agonists at GPR40 and that fatty acid-free BSA was able to inhibit the function of free fatty acids in the Ca^{2+}-mobilization assays (Fig. 2c), we explored the possibility that the high level of "basal" loading of [^{35}S]GTPγS onto GPR40-Gα_q might reflect the presence of endogenous agonist associated with GPR40 in the basal state. The most obvious means by which this could occur is via the release of free fatty acids during cell rupture and membrane preparation. Membranes of HEK293 cells transfected to express GPR40-Gα_q were incubated with or without fatty acid-free BSA (10 μM) along with either YM-254890 or GW1100. Even without these pharmacological agents, the presence of fatty acid-free BSA resulted in a large reduction in binding of [^{35}S]GTPγS in the immunoprecipitates (Fig. 4b). Furthermore, although the effect of a single concentration of GW1100 was incomplete in the absence of fatty acid-free BSA, the combination of fatty acid-free BSA and GW1100 was as effective as YM-254890 in suppressing basal binding of [^{35}S]GTPγS (Fig. 4b).

To explore the effect of fatty acid-free BSA more fully, it was added to membrane preparations in increasing concentrations, and [^{35}S]GTPγS incorporation onto GPR40-Gα_q was measured. Levels of bound [^{35}S]GTPγS decreased with increasing concentrations of BSA over the full range tested (Fig. 5) with an apparent IC_{50} value close to 1 μM. Although, as noted earlier, the stimulation of [^{35}S]GTPγS binding induced by the addition of 30 μM palmitic acid in the absence

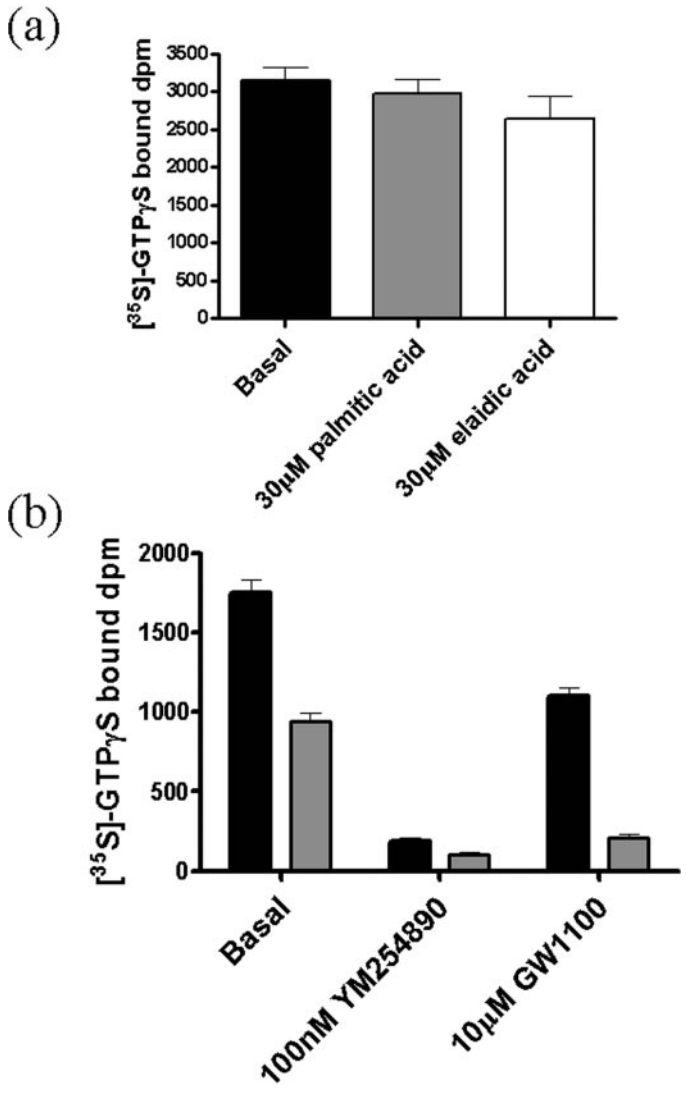

Fig. 4. GPR40 seems to display high constitutive activity in [^{35}S]GTPγS binding studies. HEK293 cells were transfected to express GPR40-Gα_q, and membranes were prepared. [^{35}S]GTPγS binding studies were then performed as described under *Materials and Methods*. a, in the absence (■) or presence of palmitic acid (▨) or elaidic acid (□) (30 μM). b, in the absence or presence of the Gα_q/Gα_{11} inhibitor YM-254890 (100 nM) or the small-molecule GPR40 blocker GW1100 (10 μM) in the absence (■) or presence (▨) of 10 μM fatty acid-free BSA.

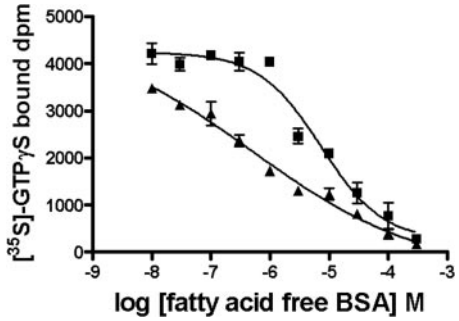

Fig. 5. Fatty acid-free BSA blocks the "constitutive" activity of GPR40. Membranes of HEK293 cells expressing GPR40-Gα_q were used in [^{35}S]GTPγS binding studies performed in the absence (▲) or presence (■) of palmitic acid (30 μM) and varying concentrations of fatty acid-free BSA. At the end of assay, GPR40-Gα_q was recovered by immunoprecipitation with the anti-Gα_q/Gα_{11} antiserum CQ (Mitchell et al., 1991), and bound [^{35}S]GTPγS was measured.

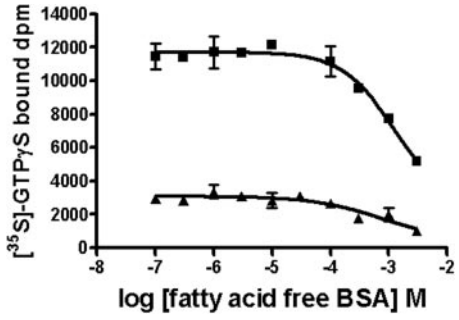

Fig. 6. GPR41 does not display high-level apparent constitutive activity that can be reversed by low concentrations of fatty acid-free BSA. HEK293 cells were transfected to express a GPR41-Gα_{i3} fusion protein, and membranes were prepared. [^{35}S]GTPγS binding studies were then performed in the absence (▲) and presence (■) of 10 mM propionic acid and the presence of varying concentrations of fatty acid-free BSA. At the end of assay, GPR41-Gα_{i3} was recovered by immunoprecipitation with a Gα_{i3}-selective antiserum (McClue et al., 1992) and counted. Data are means ± S.E.M. and are taken from a single experiment representative of three.

of fatty acid-free BSA was very limited, in the presence of concentrations of fatty acid-free BSA up to 1 μM, 30 μM palmitic acid now increased [^{35}S]GTPγS binding up to the level observed in the absence of fatty acid-free BSA (Fig. 5). At concentrations of fatty acid-free BSA between 1 and 10 μM, basal [^{35}S]GTPγS binding continued to decrease, but now 30 μM palmitic acid, although still able to stimulate [^{35}S]GTPγS binding, was unable to achieve the same maximal level (Fig. 5), potentially because substantial amounts of the added palmitic acid was now BSA-bound. These results suggest that the observed "constitutive" activity of GPR40 in the absence of fatty acid-free BSA actually reflects the presence and functional activity of an endogenous agonist associated with GPR40 that was stripped away by fatty acid-free BSA and could be competed for by GW1100. Equally, these results indicated that the use of fatty acid-free BSA could uncover a window in which the detailed pharmacology of ligands at GPR40 could be measured in [^{35}S]GTPγS binding studies.

GPR41 is closely related to GPR40 but responds only to short-chain fatty acids rather than medium- and long-chain fatty acids (Brown et al., 2003; Milligan et al., 2006). In contrast to GPR40, GPR41 couples selectively to members of the G_i family of G proteins. To explore the selectivity of the effect of fatty acid-free BSA on the apparent high-level constitutive activity of GPR40-Gα_q we constructed a GPR41-Gα_{i3} fusion protein. This construct was expressed in HEK293 cells from which membranes were prepared. We performed equivalent [^{35}S]GTPγS binding studies but now recovered this construct via immunoprecipitation with a Gα_{i3}-selective antiserum (McClue et al., 1992). Basal [^{35}S]GTPγS binding was low and increased some 4- to 5-fold with the addition of the short-chain fatty acid propionate (Fig. 6). Basal [^{35}S]GTPγS binding in such immunoprecipitates was entirely unaffected by the addition of fatty acid-free BSA until the concentration was increased beyond 100 μM (Fig. 6), and this was also true for the agonist function of propionate (Fig. 6). As such, not all of the GPR40–43 family of free fatty acid receptors display the apparent high level constitutive activity in [^{35}S]GTPγS binding studies that was observed for GPR40 and the effect of fatty acid-free BSA to reduce the "apparent" constitutive activity of GPR40 at concentrations lower than 100 μM is not a nonspecific artifact.

To begin to explore the pharmacology of GPR40 in [^{35}S]GTPγS binding studies, we measured binding of [^{35}S]GTPγS in HEK293 cell membranes expressing GPR40-Gα_q in the presence of increasing concentrations of palmitic acid and in the presence of three concentrations of fatty acid-free BSA (Fig. 7). In the presence of a low concentration of fatty acid-free BSA (100 nM), as described earlier, basal [^{35}S]GTPγS binding to GPR40-Gα_q was high and essentially not modulated by palmitate. In contrast, in the presence of either 10 or 100 μM fatty acid-free BSA, basal [^{35}S]GTPγS binding was greatly reduced, hence allowing the concentration-response curves to palmitic acid to be established (Fig. 7). At the higher concentration of fatty acid-free BSA, basal

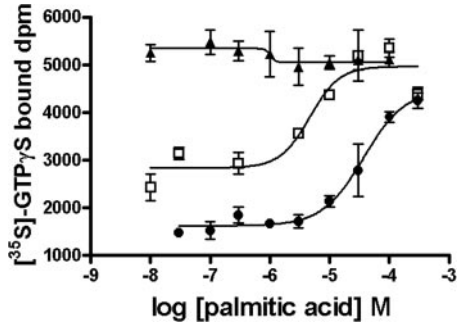

Fig. 7. Fatty acid-free BSA uncovers the pharmacology of GPR40. Membranes of HEK293 cells expressing GPR40-Gα_q were used in [^{35}S]GTPγS binding studies as in Fig. 5 performed in the presence of varying concentration of palmitic acid and differing concentrations [(▲) 100 nM, (□) 10 μM, (●) and 100 μM] of fatty acid-free BSA.

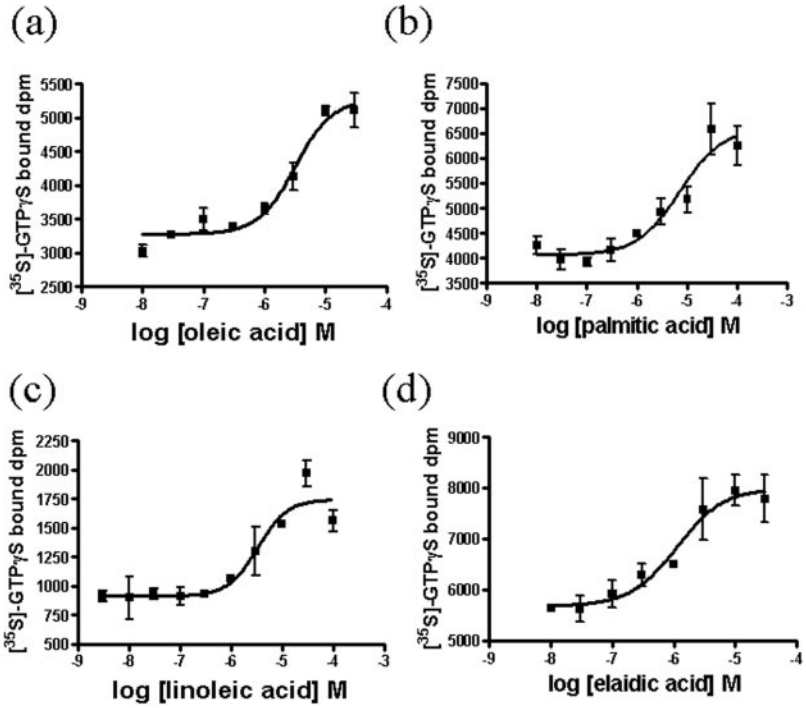

Fig. 8. GPR40-Gα_q can be activated by various fatty acids. The pharmacology of activation of GPR40-Gα_q by oleic acid (a), palmitic acid (b), linoleic acid (c), and elaidic acid (d) was examined via [^{35}S]GTPγS binding studies in membranes of HEK293 cells transiently transfected to express this construct. The apparent high constitutive activity of GPR40-Gα_q was reduced by treatment with fatty acid-free BSA (10 μM).

[^{35}S]GTPγS binding was more substantially reduced, and the concentration-response curve to palmitic acid was shifted to higher apparent concentrations (pEC_{50} = −4.4 with 10 μM BSA and −3.7 with 100 μM BSA) (Fig. 7). This is again consistent with the higher amount of fatty acid-free BSA binding a greater amount of the added palmitic acid. Using this fatty acid-free BSA "stripping" approach, it was then possible to examine the pharmacology and potency of a range of GPR40 agonists, including other fatty acids (Fig. 8), thiazolidinediones (Fig. 9a), and the small-molecule agonist GSK250089A (structure 14B in Garrido et al., 2006) (Fig. 9b) that is a close structural analog of the small-molecule GPR40 agonist GW508 studied by Briscoe et al. (2006).

Even in the presence of 10 μM BSA, "basal" [^{35}S]GTPγS binding was substantially further reduced by GW1100 in a concentration-dependent manner (Fig. 10), suggesting either that GW1100 is indeed a GPR40 inverse agonist or that GW1100 was able to compete with remaining endogenous agonist that was not removed by the fatty acid-free BSA treatment. If the second explanation is correct, then GPR40 has only low true constitutive activity. In the presence of 10 μM BSA, the reduction of basal [^{35}S]GTPγS binding allowed direct detection of the agonist activity of GSK250089A (Fig. 11a). However, in the absence of BSA, the agonist activity of GSK250089A could only be detected in the presence of GW1100 (Fig. 11b) because this reduced the basal [^{35}S]GTPγS signal (Fig. 11, a and b). It is interesting that when concentration-response curves of the ability of GSK250089A to activate GPR40-Gα_q were performed in the presence of 10 μM GW1100 to reduce the apparent basal [^{35}S]GTPγS binding, even very high concentrations of GSK250089A were unable to stimulate [^{35}S]GTPγS binding to the levels observed in the absence of GW1100 (Fig. 11c). These data indicate that GSK250089A is a partial agonist at GPR40-Gα_q. In a similar fashion, the presence of either fatty acid-free BSA or GW1100 allowed 1 μM troglitazone to elevate [^{35}S]GTPγS binding to GPR40-Gα_q (Fig. 11d), and GW1100 inhibited this in a concentration-dependent fashion (Fig. 11e).

Because all of the above studies were performed on cells and cell membrane preparations into which forms of GPR40 has been introduced by transfection, we wished to assess whether similar results would be obtained in cells that endogenously express GPR40. Human pancreatic β-cell lines are not generally available. However, rat insulinoma INS-1E cells have been used widely to explore many aspects of the regulation of insulin release and the contribution of GPR40 (Shapiro et al., 2005). We confirmed expression of GPR40 and the housekeeping gene cyclophilin in INS-1E cells via reverse transcription-polymerase chain reaction (Fig. 12a) and that both troglitazone and free fatty acids, including lauric acid, generated a robust and transient elevation of $[Ca^{2+}]_i$ in these cells (Fig. 12b). After membrane preparation, we performed a series of [^{35}S]GTPγS binding studies. With end-of-assay immunoprecipitation of Gα_q, levels of bound [^{35}S]GTPγS were again high in the absence of added ligands (Fig. 12c), and little stimulation was produced by the addition of free fatty acids (data not shown). As with HEK293 cell membranes, the addition of fatty acid-free BSA resulted in a large reduction in "basal" [^{35}S]GTPγS in Gα_q immunoprecipitates, and these levels were now increased markedly by fatty acids, including palmitic acid (Fig. 12c). However, in contrast to the HEK293 cell studies, in which we introduced forms of human GPR40, in INS-1E cell membranes, the effect of troglitazone, although statistically significant, was not as robust as that produced by palmitic acid (Fig. 12c). It is interesting that, in the murine pancreatic β-cell line MIN6, rosiglitazone also seems to act as a partial agonist at GPR40 (Kotarsky et al., 2003a).

Discussion

The GPR40–43 family of free fatty acid receptors has attracted a great deal of attention in recent years (Brown et al., 2005; Milligan et al., 2006), not least because of the expression of GPR40 in pancreatic β-cells and the ability of agonists to function as glucose concentration-dependent in-

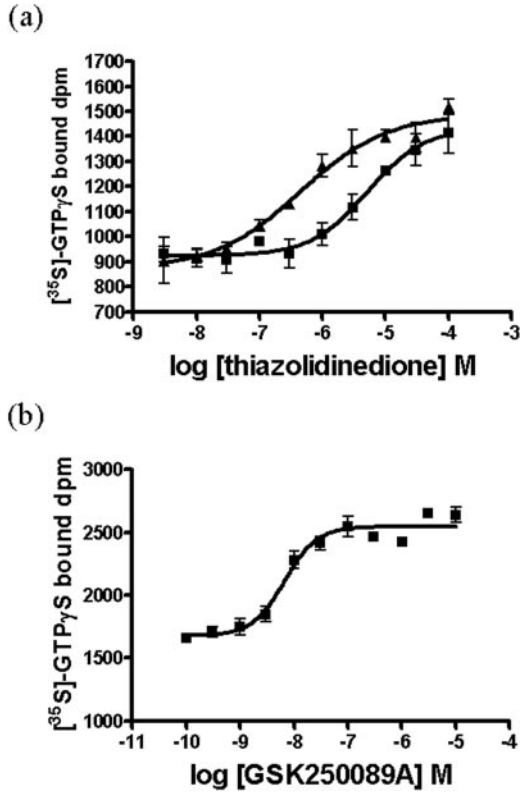

Fig. 9. Thiazolidinediones and the small-molecule GSK250089A are potent agonists at human GPR40. The ability of various concentrations of rosiglitazone (a, ■), troglitazone (a, ▲), and GSK250089A (b) to promote binding of [^{35}S]GTPγS to GPR40-Gα_q was assessed as in Fig. 8.

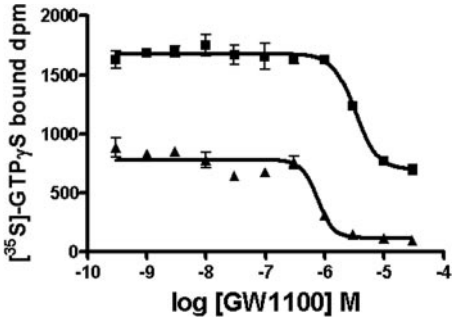

Fig. 10. Pharmacology of GW1100 at GPR40. HEK293 cell membranes expressing GPR40-Gα_q were used in [^{35}S]GTPγS binding assays performed in the presence of varying concentrations of GW1100 and the absence (■) or presence (▲) of 10 μM fatty acid-free BSA.

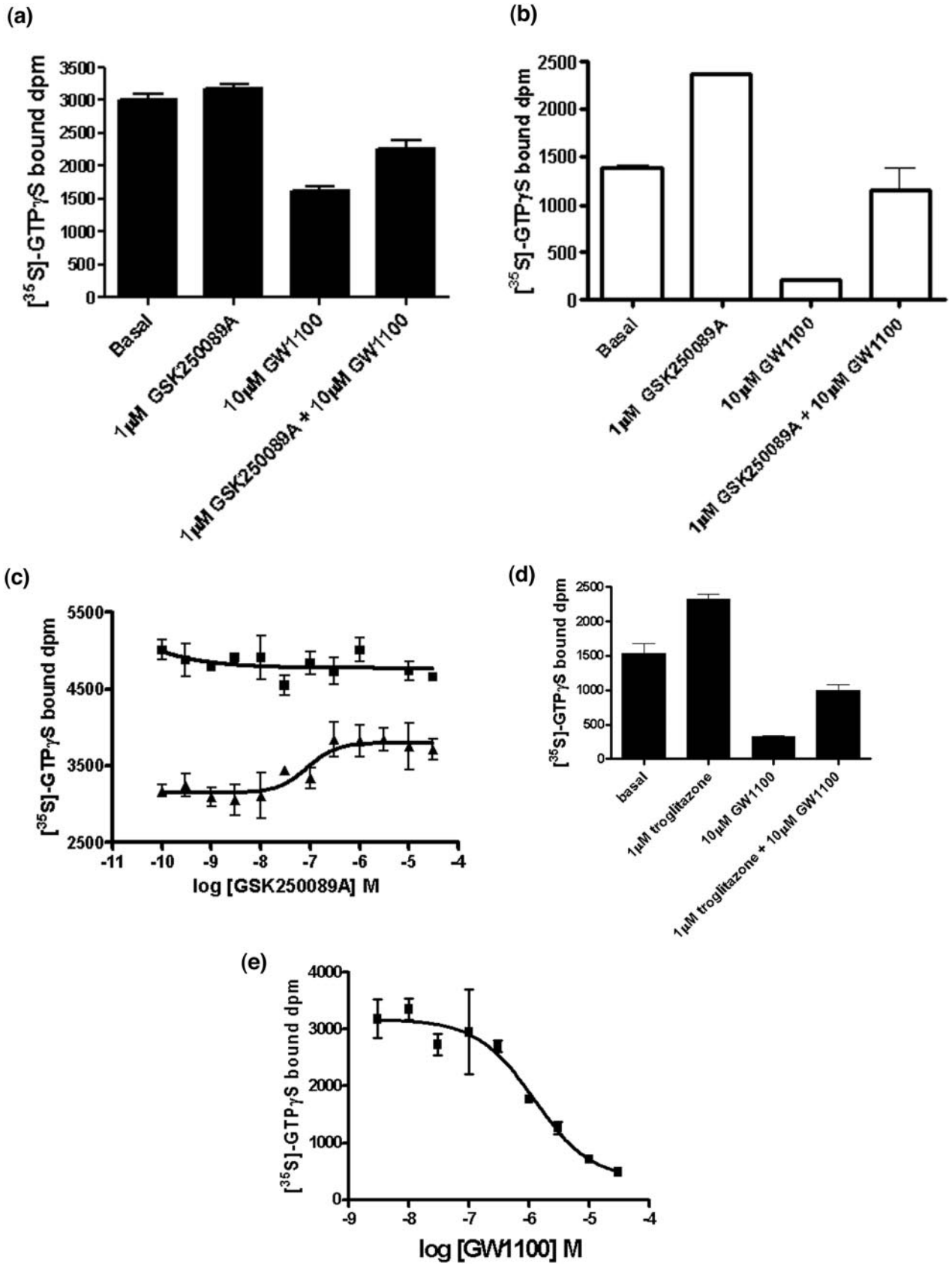

Fig. 11. Use of GW1100 to uncover the pharmacology of human GPR40. HEK293 cell membranes expressing GPR40-$G\alpha_q$ were used in [^{35}S]GTPγS binding assays in the presence (a) or absence (b) of 10 μM fatty acid-free BSA. Samples also contained 1 μM GSK250089A, 10 μM GW1100, or both ligands. c, [^{35}S]GTPγS binding in response to varying concentrations of GSK250089A were measured in the absence (■) and presence (▲) of 10 μM GW1100. The effect of 1 μM troglitazone was assessed in the presence 10 μM fatty acid-free BSA with or without 10 μM GW1100 (d) or varying concentrations of GW1100 (e).

sulin secretagogues. Agonist ligands act to stabilize and enrich populations of active states of GPCRs (Milligan, 2004). Although 11-cis retinal is bound by opsins as an endogenous inverse agonist to virtually eliminate active states in the absence of light, other GPCRs display varying levels of agonist-independent or constitutive activity (Milligan, 2004; Bond and IJzerman, 2006). At least in transfected cell systems this can be extensive and may virtually occlude the detection of effects of agonists above such basal activity (Canals et al., 2006). Although views of the importance of constitutive activity of GPCRs in native tissues have waxed and waned in recent years, there is significant evidence that this may be relevant to physiology and, potentially, to the effects of therapeutic medicines (Bond and IJzerman, 2006). However, a question that has always dogged interpretation of constitutive activity and consideration of whether "inverse" agonists might have inherent advantages as medicines is that it has been difficult to eliminate the possibility that "basal" activity might actually reflect the presence of an endogenous agonist that is difficult to eliminate or remove.

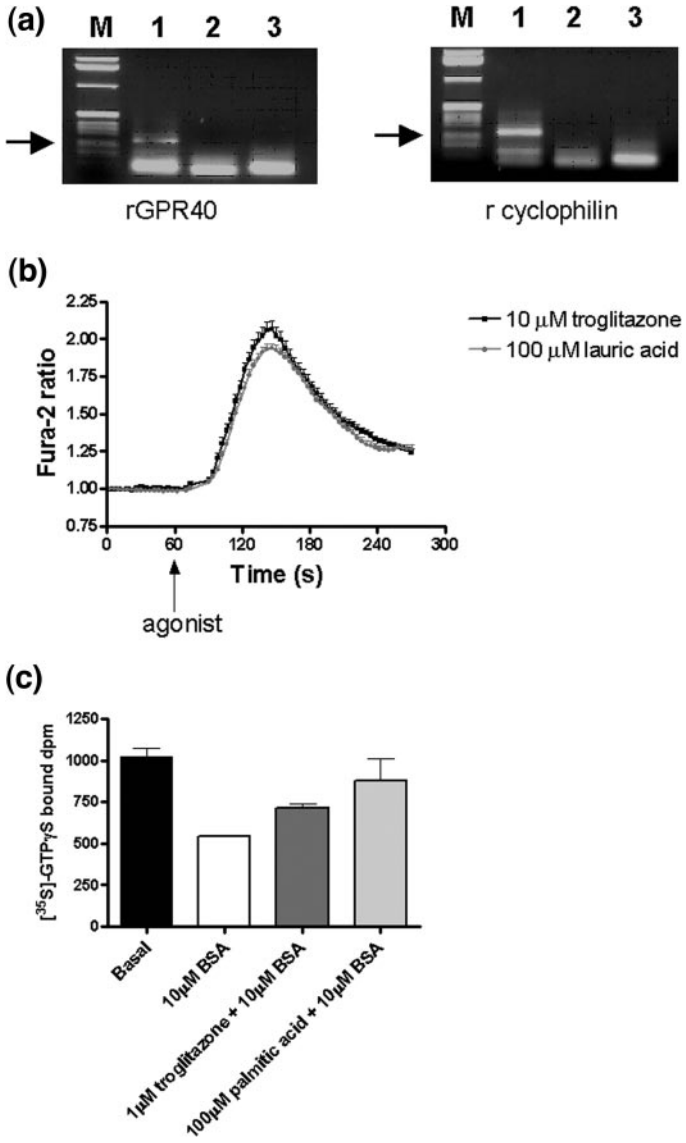

Fig. 12. Treatment with fatty acid-free BSA reduces apparent high constitutive activity in INS-IE cells that express GPR40 endogenously. a, mRNA isolated from rat INS-IE cells was reverse-transcribed to generate cDNA that was used in PCR studies to detect messages corresponding to GPR40 (left) and cyclophilin (right). M, size markers; 1, plus cDNA; 2, minus cDNA; 3, minus reverse transcriptase. b, after loading of INS-IE cells with Fura-2, 10 μM troglitazone (black), or 100 μM lauric acid (gray) were added at the 60-s time point, and the alteration in Fura-2 fluorescence was monitored over time as described under *Materials and Methods*. Data represent means ± S.E.M. from 20 individual cells. c, membranes were prepared from INS-IE cells. Basal (black bar) binding of [^{35}S]GTPγS in Gα_q immunoprecipitates, and the effects of 10 μM fatty acid-free BSA alone (white bar) and 10 μM fatty acid-free BSA plus troglitazone (dark gray bar) or plus palmitic acid (light gray bar) on levels of [^{35}S]GTPγS bound were assessed.

It is now well appreciated that a substantial number of GPCRs respond to hydrophobic, lipid, or lipid-like endogenous mediators. These may be particularly difficult to remove if they are long-lived. As such, when developing the [^{35}S]GTPγS binding studies herein, detection of very high levels of "constitutive" activity were of considerable interest not only because of the hydrophobic nature of the endogenous agonists at GPR40 but also because high-level constitutive activity has been reported for other GPCRs that are of therapeutic interest in the areas of feeding, obesity, and the metabolic syndrome, such as the ghrelin receptor (Holst and Schwartz, 2004; Holst et al., 2004). Although it might be argued that the apparent high constitutive activity of GPR40 in these assays could relate to the close proximity enforced between GPCR and G protein by the use of a GPR40-Gα_q fusion protein, this is unlikely because we have not observed this characteristic with other GPCR-Gα_q fusions we have studied previously (Carrillo et al., 2002; Milligan et al., 2004).

A key issue in these studies is that high basal activity of GPR40 was only detected using [^{35}S]GTPγS binding assays and not in Ca^{2+}-mobilization assays. As discussed earlier, Ca^{2+} mobilization assays are inherently unsuited to the detection of receptor constitutive activity because of the capacity and requirement of intact cells to dynamically re-equilibrate alterations in Ca^{2+} levels. The potential key difference in the two assays is that the [^{35}S]GTPγS binding assay used membrane preparations. Although it must remain speculative, some of the fatty acids released upon cell breakage might be expected to remain in the membranes and potentially be bound to GPR40 during preparation rather than partitioning into the wash buffer. In an attempt to assess whether members of the GPR40–43 group of receptors that respond to short-chain fatty acids would also display high apparent constitutive activity that could be reversed by treatment with fatty acid-free albumin, we generated and expressed a GPR41-Gα_{i3} fusion protein. This showed relatively low levels of basal [^{35}S]GTPγS binding, which were increased markedly in the presence of the agonist propionate. However, unlike GPR40-Gα_q, at concentrations up to 100 μM, fatty acid-free BSA was unable to reduce basal [^{35}S]GTPγS binding to the GPR41-Gα_{i3} construct. Gα_i-family G proteins generally have substantially higher levels of spontaneous, ligand, and GPCR-independent guanine nucleotide exchange than Gα_q family G proteins, and this is likely to account for the basal [^{35}S]GTPγS binding observed. Even if short-chain fatty acids were also released by cell breakage, these are relatively water soluble and would probably be washed out during membrane preparation.

Because the majority of "free" fatty acids in serum are complexed with albumin and other proteins, we interpret the ability of fatty acid-free BSA to reduce the basal binding of

[^{35}S]GTPγS to GPR40-Gα_q as an indication that it removed unappreciated fatty acids from the receptor. In favor of this model, although the addition of palmitic or lauric acid was unable to significantly increase binding of [^{35}S]GTPγS without the addition of fatty acid-free BSA, the effect of these agents in combination with fatty acid-free BSA treatment was to elevate [^{35}S]GTPγS binding back to the basal levels. As anticipated, this only occurred over a limited range of fatty acid-free BSA concentrations. Although increasing this further continued to decrease basal levels, the effects of the added fatty acids were eventually decreased, presumably as the added fatty acid also became bound to BSA. Perhaps most convincingly in this regard, the observed concentration-response curve for palmitic acid moved to higher added concentrations as greater concentrations of BSA were added to further reduce basal [^{35}S]GTPγS binding.

The effect of the GPR40 blocker GW1100 (Briscoe et al., 2006) was also to reduce basal [^{35}S]GTPγS binding to GPR40-Gα_q. Although the conventional (and most obvious) interpretation of this effect would be to describe GW1100 as an "inverse" agonist at GPR40, it was not possible to assess clearly whether this might indeed reflect suppression of a high level of constitutive activity or competition with an endogenous agonist to bind the receptor. This type of dilemma can be resolved if both inverse agonists and neutral antagonists are available for a GPCR. However, for recently characterized GPCRs with limited small-molecule pharmacology, this poses considerable challenges and has been the most limiting issue in defining the contribution of constitutive activity to the physiological function of GPCRs (Gardner and Mallet, 2006; Negus, 2006). It is thus sensible to simply refer to GW1100 as a GPR40 antagonist until this issue can be clearly defined.

The use of fatty acid-free BSA also provided an excellent window via which the pharmacology of GPR40 to activate Gα_q could be monitored no matter whether using fatty acids, novel small molecules, or the insulin-sensitizing, PPARγ-agonistic thiazolidinediones. The use of [^{35}S]GTPγS binding studies rather than $[Ca^{2+}]_i$ measurements also allowed GSK250089A to be defined as a partial agonist at GPR40. Maximally effective concentrations of this ligand were unable to stimulate binding of [^{35}S]GTPγS to GPR40-Gα_q to the same level as observed for the apparent basal activity.

The observations that therapeutically useful thiazolidinediones, such as rosiglitazone and troglitazone, act as agonists at GPR40 confirm the observations of Kotarsky et al. (2003a,b) and Milligan et al. (2006) and pose interesting questions as to possible contributions of GPR40, rather than the PPARγ isoforms, to their actions. This is particularly true because, although there is good correlation between the EC_{50} for PPARγ agonism and the in vivo minimum effective dose of a range of thiazolidinediones (Wilson et al., 1996), the EC_{50} values are also very similar to those we record here for GPR40 agonism. Furthermore, because a series of rapid, apparently nongenomic, effects of thiazolidinediones have been reported (Gardner et al., 2005), these may reflect activation of GPR40. The recent development of GPR40 knockout animals (Steneberg et al., 2005) and both further studies with selective knockdown of GPR40 in model systems and a detailed analysis of the overlap and separation of GPR40 and PPARγ pharmacology will contribute to this important question. Of considerable interest in this regard is the structure-activity relationship of the thiazolidinediones. Troglitazone was more potent than rosiglitazone at human GPR40, whereas pioglitazone and rosiglitazone are at least 100-fold more potent than troglitazone at PPARγ.

Finally, the detection of an apparent high level of GPR40 constitutive activity and the reversal of this by the addition of fatty acid-free BSA was not restricted to transfected cells. Equivalent observations were made using the rat pancreatic β-cell line INS-1E that expresses this receptor endogenously and that is widely used as a model system to examine the regulation of insulin secretion. These studies suggest that careful consideration needs to be given to the basis of high levels of measured constitutive activity for both orphan GPCRs and recently deorphanized GPCRs for which limited small-molecule pharmacology is available.

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