Mouse GPR40 heterologously expressed in Xenopus oocytes is activated by short-, medium-, and long-chain fatty acids

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G PROTEIN-COUPLED RECEPTORS (GPCRs) play a vital signaling role in numerous biological processes, with an ever-increasing number of endogenous physiological ligands. These include lipid mediators, such as leukotrienes and prostaglandins. Recent attention has turned to the possibility of GPCRs sensing exogenous ligands, including nutrients. High throughput screening and ligand fishing in systems which heterologously express orphan GPCRs has recently assigned four receptors with unknown ligands as receptors for fatty acids. These are denoted GPR40, GPR41, GPR43, and GPR120. The limited published data reported that both GPR40 (11) and GPR120 (10) specifically detect long-chain fatty acids, with 12 or more carbon atoms (C12:0) required for signaling activity from GPR40 expressed in Chinese hamster ovary cells and from GPR120 expressed in human embryonic kidney (HEK)-293 cells, respectively. However, GPR40 was also reported to respond to a medium-chain, (C6:0) but not a short-chain (C1), fatty acid when expressed in HEK-293 cells (1). Therefore the true chain length specificity of GPR40 remains uncertain. In contrast, GPR41 and GPR43 are reportedly stimulated only by short-chain fatty acids (C1-C6:0), sharply becoming far less responsive to fatty acids of seven or more carbons (2, 14).

The functional evidence is strongest that GPR40 acts as the receptor for fatty acid-induced insulin secretion. It is abundantly expressed in pancreatic β-cells (11), and GPR40 knockout and overexpression in vivo have now been shown to mediate both acute and chronic effects of long-chain free fatty acids on murine insulin secretion (22). These data suggest that GPR40 may form a mechanistic link between diet, obesity, and Type 2 diabetes (22). Increased pancreatic expression of GPR40 mRNA is observed in obese mice that lack leptin (13), whereas polymorphisms so far detected in the human GPR40 gene are not associated with abnormal insulin release (7).

Activation of GPR40 is coupled to an increase in intracellular Ca²⁺ concentration ([Ca²⁺]i) (1, 11, 12, 20). In addition, it has already been reported that anti-diabetic drugs (e.g., thiazolidinediones) and experimental anti-obesity drugs (e.g., MEDICA 16) activate GPR40-expressing cells and the pancreatic β-cell line MIN6 (12). GPR40 has also been detected in the MCF-7 human breast cell line (25) and has been implicated in control of breast cancer cell growth by fatty acids (8). The other members of this group of receptors are less well characterized functionally. GPR120 promotes the secretion of the glucagon-like peptide-1 from enteroendocrine L-cells (10). GPR41 plays a role in leptin production in adipocytes (24), whereas GPR43 is implicated in differentiation and immune responses of monocytes and granulocytes (19).

The presence of GPR40 mRNA has also been reported in the small and large intestine (1, 11), and in the fatty acid-responsive intestinal enteroendocrine cell line STC-1 (10). We have previously reported (9) that STC-1 cells respond to fatty acids via mobilization of [Ca²⁺]i, in a chain length-dependent pattern requiring 12 or more carbon atoms. This suggests a possible role for GPR40 in fatty-acid sensing by STC-1 cells. However, the effects of fatty acids are complex, and our recent data have demonstrated that fatty acids also exert a receptor-independent effect to mobilize [Ca²⁺]i, directly from endoplasmic reticulum stores in STC-1 cells (9). In addition, STC-1 cells also express GPR120, making it impracticable to fully characterize the role of GPR40 alone in fatty acid sensing in this multimodal cell line.
Therefore, the aim of the current study was to heterologously express in *Xenopus laevis* oocytes mouse GPR40 cDNA isolated from STC-1 cells and further clarify the fatty acid chain length specificity of GPR40. In contrast to other reports of GPR40 function, we found that GPR40 is stimulated by fatty acids of all chain length groupings, i.e., short, medium, and long. This new finding has important implications for understanding fatty acid chain length-dependent processes that involve GPR40, and calls for caution in ascribing overly prescriptive functional nomenclature to such recent orphans.

**MATERIALS AND METHODS**

**RT-PCR.** Poly-A RNA was isolated by guanidine/thiocyanate extraction from STC-1 cells cultured in 75-cm² tissue culture flasks. cDNA was prepared from 1 µg poly-A RNA using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA), and subjected to PCR using primers based on the mouse GPR40 mRNA sequence (GenBank Accession No. NM194057). Full-length mGPR40 primers were synthesized (MWG Biotech) with added restriction enzyme sites XhoI for 5′ and SacII for 3′ ligations (forward primer: 5′-CTCGAGATGatgacctgcccccacagttc-3′, reverse primer: 5′-CCGCGGctgtctgaattgttcctcTTTGAGT-3′). PCR conditions were 94°C for 2 min, then 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. PCR products were separated by 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining.

**Cloning and sequencing.** The 903-bp product was extracted using QIAquick gel extraction kit (Qiagen) and inserted into the Invitrogen cloning vector pCR4-TOPO. This construct was transformed into *Escherichia coli* and selected colonies cultured. The plasmid cDNA was isolated using QIAprep spin miniprep kit (Qiagen), and the cDNA product sequenced (Lark Technologies, Essex, UK).

**Oocyte expression.** Female *Xenopus laevis* frogs were anesthetized using 0.2% tricaine, euthanized by decapitation in accordance with UK Home Office regulations, and their oocytes were then excised. The oocytes were treated with 2 mg/ml collagenase (Sigma) in Ca²⁺-free Barth’s solution for 1 h. Collagenase-defolliculated stage V/VI oocytes were injected with 5–10 ng of mGPR40 cRNA. The cRNA was produced from mGPR40 cDNA and subcloned into the oocyte expression vector PT7TS. Oocytes were incubated for 1–3 days at 18°C. Activation of mGPR40 receptors expressed in oocytes would be anticipated to increase [Ca²⁺⁺], in turn stimulating the endogenous biphasic, Ca²⁺⁺-activated chloride current (CaCC) (3) that is sensitive to changes in external pH (18) and to the stilbenedi-sulfonate 4,4-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) (23). This chloride current was measured using two-electrode voltage-clamp recordings. Recordings were made using the two-electrode voltage-clamp method, with oocytes clamped at −60 mV with a Warner OC-725C amplifier. Oocytes were perfused in a standard ND96 bath solution composed of (in mM) 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, and 5 HEPES (pH 7.4). Fatty acid solutions were diluted from 500 mM stock solutions, sonicated for 10 min (Soniprep 150, Sanyo), and then applied at a close-range superfusion rate of 1.5

![Fig. 1. Enteroendocrine cell line (STC-1) cells express G protein coupled receptor 40 (GPR40). Specific mouse GPR40 primers detected the expected 903 bp RT-PCR product in cDNA derived from STC-1 mRNA. +, mouse GPR40 cDNA positive control; −, negative control of STC-1 mRNA without RT reaction; STC-1, STC-1 mRNA with RT reaction.](image)

![Fig. 2. Linoleic acid stimulates Ca²⁺⁺-activated chloride currents in mGPR40-injected oocytes. A: two-electrode voltage clamp recordings, during 2-min exposures to 500 µM linoleic acid (C18:2), showing a current is stimulated in mGPR40-injected oocytes but not water-injected oocytes. B: the linoleic acid stimulated current is biphasic, with an initial peak phase, followed by a prolonged plateau phase that remains while fatty acid is present (i.e., up to 2 min). C: summary of the significant peak current (n = 22, **P < 0.01, ANOVA) and the significant plateau current that remains at the end of 2 min of linoleic acid exposure (n = 22, *P < 0.05, ANOVA).](image)
ml/min for 2 min from a capillary 2 mm from the oocyte. Currents were recorded and peak currents measured using pCLAMP8 software (Axon Instruments).

Data analysis. Changes in current were presented as means ± SE. Statistical analysis of oocyte currents was performed using one-way ANOVA or the unpaired t-test as appropriate, using the GraphPad Instat software package. For ANOVA, Student-Newman-Keuls post hoc tests were performed and significant differences determined at a 5% level.

RESULTS

STC-1 cells express GPR40 mRNA. RT-PCR was performed using specific mouse GPR40 primers and the expected 903-bp signal was detected in STC-1 cells (Fig. 1). The PCR product was excised from the gel and cloned into a plasmid vector for DNA sequencing analysis. Although a single nucleotide polymorphism occurred at base 399 from the start codon compared with the published sequence of mouse GPR40 (Accession No. AF539809), this was a silent mutation because the encoded amino acid sequence was not altered.

Xenopus oocyte expression of GPR40 confers fatty acid responsiveness. During two-electrode voltage-clamp experiments, the addition of linoleic acid (C18:2) significantly stimulated a current in mGPR40-injected oocytes, but not in water-injected controls (Fig. 2A). The peak change in current with 500 μM linoleic acid for water-injected controls was −0.01 ± 0.01 μA (n = 12), but for mGPR40-injected oocytes was −0.44 ± 0.07 μA (n = 22) (P < 0.01, ANOVA). Several experiments were performed to confirm that the linoleic acid-stimulated currents observed were indeed Ca2+-activated chloride currents (CaCC). As would be expected of the endogenous Xenopus CaCCs, the stimulated currents were biphasic (3), with an initial peak phase, followed by a prolonged plateau phase that remained while fatty acid was present (Fig. 2B). Initial peak responses occurred within 1 min (51 ± 8 s), whereas complete recovery from the plateau phase took ~5 min during washout (314 ± 53 s). Interestingly, the final 0.02–0.03 μA of full recovery occupied the last 1 to 2 min of the process, probably reflecting the fact that cell-associated fatty acids cannot be washed out fully in a short time. Compared with initial values of −0.02 ± 0.01 μA (n = 22), currents were still significantly activated after 2-min exposure to 500 μM linoleic acid at −0.13 ± 0.03 μA (n = 22, P < 0.05, ANOVA) (Fig. 2C). However, on removal of linoleic acid, currents returned to basal levels of −0.03 ± 0.01 μA (n = 22). To further confirm that the stimulated current was the pH-sensitive CaCC (17), the effect of reducing external pH was investigated (Fig. 2D). In experiments performed with a bath solution at pH 5.5 (pH 7.4), linoleic acid remained ineffective on water-injected oocytes (NS, ANOVA). However, the currents stimulated by 500 μM linoleic acid in mGPR40-injected oocytes were significantly attenuated at pH 5.5 (−0.04 ± 0.02 μA; n = 4) compared with pH 7.4 (−0.25 ± 0.07 μA; n = 4) (P < 0.01, ANOVA). In addition, the linoleic acid-stimulated current required ~25 min to fully recover, similar to the recovery time period previously reported for endogenous CaCCs in Xenopus oocytes (3). This was demonstrated by the reduced response seen if a second application of linoleic acid occurred just 10 min after the first (P <

Fig. 2—Continued. D: reduction of external pH from 7.4 to 5.5 significantly reduced the current stimulated by 500 μM linoleic acid (P < 0.01, ANOVA) in mGPR40-injected oocytes, without having an effect on water-injected oocytes (ns, ANOVA). E: the linoleic acid-stimulated current requires a significant recovery period before a full response can be initiated again. Measured as a percentage of the initial response, the size of a second response is significantly less if the washout period is 10 min compared with 25 min (P < 0.05, unpaired t-test). The linoleic acid stimulated current is also significantly inhibited by the presence of 100 μM DIDS (P < 0.05, unpaired t-test) (F) or 0.1% BSA (P < 0.05, unpaired t-test) (G). *P < 0.05; **P < 0.01.
0.05, unpaired t-test), compared with a full second response seen after 25-min recovery (Fig. 2E). The linoleic acid stimulated current was also significantly inhibited by 100 μM DIDS (P < 0.05, unpaired t-test) (Fig. 2F), an inhibitor of anion transport known to inhibit CaCCs (23). Finally, we investigated the effect of adding 0.1% bovine serum albumin (BSA), which avidly binds and hence removes >99% of any free fatty acids. BSA has previously been shown to inhibit GPR40-induced Ca²⁺ responses (11). The addition of 0.1% BSA drastically inhibits the linoleic acid current (Fig. 2G), showing as expected that the stimulated GPR40-induced current is dependent on free fatty acid concentration.

**Chain length and dose dependency of GPR40 activation by fatty acids.** In two more electrode voltage-clamp experiments, the addition of the short-chain fatty acid butyric acid (C4:0) significantly stimulated a current in mGPR40-injected oocytes, but not water-injected controls (Fig. 3A). The mean peak currents with 500 μM butyric acid for water-injected oocytes were 0.00 ± 0.01 (n = 4) compared with the significant currents of −0.55 ± 0.10 (n = 13, P < 0.01, ANOVA) observed in mGPR40-injected oocytes. This finding is in contrast with the previous report (11) that found mGPR40 insensitive to this short-chain fatty acid, whereas Briscoe et al. (1) stated that the minimum chain length requirement to be effective was C6:0. The butyric acid stimulated current, similar to that observed with linoleic acid, was again biphasic (Fig. 3B), with a significant plateau current of −0.15 ± 0.03 (n = 13, P < 0.05; ANOVA) compared with control and washout values of −0.03 ± 0.01 (n = 13) (Fig. 3C). Finally, to further confirm that the butyric acid stimulated currents were the same currents as those stimulated by linoleic acid, we investigated the effect of BSA. As predicted, the addition of 0.1% BSA almost completely inhibited the peak current observed with 500 μM butyric acid (P < 0.05, unpaired t-test) (Fig. 3D).

Because a short-chain fatty acid such as butyric acid had been shown to stimulate mGPR40-injected oocytes, a wide range of other fatty acids were also investigated using two electrode voltage clamping. In terms of other short-chain fatty acids, acetic acid (C2:0) had no effect at a concentration of 500 μM, with a peak current value of 0.01 ± 0.01 μA (n = 4, NS, ANOVA), and neither did 500 μM propionic acid (C3:0) (0.00 ± 0.02, n = 4, NS, ANOVA). In contrast, both hexanoic acid (C6:0) and caprylic acid (C8:0) stimulated currents comparable with those observed with linoleic acid (data not shown).
shown). Medium-chain fatty acids also stimulated significant currents at 500 μM ($P < 0.01$, ANOVA): capric acid (C10:0) $-0.48 \pm 0.10 \mu$A ($n = 4$); lauric acid (C12:0) $-1.14 \pm 0.34 \mu$A ($n = 5$). Finally, the long-chain fatty acid, oleic acid (C18:1), also stimulated significant currents (data not shown). These results confirm that a wide range of fatty acid chain lengths stimulate mGPR40 when expressed in oocytes. The magnitude of the stimulated current was not dependent on fatty acid chain length (data not shown). Importantly, in direct control experiments performed on the same day, no currents were ever stimulated in water-injected oocytes (data not shown). In addition, 10 mM glucose had no effect on mGPR40-specific response similar in size to CoA alone (data not shown, NS, ANOVA), but smaller than that obtained with 250 μM of the corresponding free fatty acid (data not shown, $P < 0.05$, ANOVA). Finally, we investigated the effect of adding BSA on the CoA response, along with responses to 500 μM lauric acid and 2.5 μM ionomycin (Fig. 5B). For both CoA and lauric acid, the addition of 0.1% BSA almost completely abolished the response ($P < 0.01$, ANOVA). In contrast, the CaCC conductance stimulated by 2.5 μM ionomycin, a $Ca^{2+}$ ionophore, was unaffected by BSA addition (Fig. 5B) (NS, ANOVA).

**DISCUSSION**

GPR40 has been assigned as a long-chain (11) or a medium- and long-chain (1) fatty acid receptor, with most subsequent research linking it to fatty acid-induced insulin secretion by pancreatic β-cell (11, 20, 22). However, the fact that the precise fatty acid ligands activating GPR40 differed in the two available reports (1, 11) means that we do not yet understand its most fundamental characteristics. This requires further elucidation, as does its roles outside the β-cell. In this study, we present several novel observations about the fatty acid sensing profile of GPR40.

Initially, we confirmed the presence of mouse GPR40 mRNA in STC-1 cells by RT-PCR. Heterologous expression of mGPR40 cRNA into *Xenopus* oocytes conferred sensitivity to

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**Fig. 4.** The dose-dependent effects of various fatty acids on mGPR40-injected oocytes. Dose response curves are shown for butyric acid (C4:0) (A), capric acid (C10:0) (B), lauric acid (C12:0) (C), and linoleic acid (C18:2) (D). Oocytes were exposed to a range of fatty acid concentrations, from 1 to 1,000 μM. Typical responses to 10, 100, 500, and 1,000 μM are shown for each fatty acid because no current stimulation was ever obtained at the 1 μM level. Normalized response curves plotted against log of fatty acid concentration are also shown.
fatty acids, such as lauric acid (C12) and linoleic acid (C18:2), as predicted. Nonspecific stimulatory effects on oocytes were carefully excluded by using water-injected controls for each fatty acid. The activation of mGPR40 receptors expressed in the oocytes caused an increase in \([\text{Ca}^{2+}]_i\) because this activated the endogenous CACC measured. The stimulated current was confirmed as CACC by several experiments. First, the predicted biphasic nature of the current (3) was clearly identified (Fig. 2, A–C) and the sensitivity to changes in external pH (18) was then confirmed (Fig. 2D). In addition, delayed recovery of the current during repeated exposures (3) and sensitivity to the chloride channel blocker DIDS (23) were also confirmed (Fig. 3, E and F, respectively).

Intriguingly, the fact that mGPR40 expressed in oocytes also responds to a key short chain fatty acid, butyric acid (C4:0), extends its potential functionality beyond the more restrictive chain-length specificity previously reported for GPR40 (1, 11). Although we employed fatty acids at concentrations up to 1,000 \(\mu\)M, which were higher than those used in previous studies (1, 11), these were still well within the physiological range encountered for fatty acids in the gut. This is discussed in detail below. Our data therefore suggest that the longer acyl chain selectivity previously described is a consequence of not employing fatty acids at higher concentrations. However, we did not find an effect with even shorter fatty acids, i.e., C2 or C3. When analyzed in mammalian cells, GPR40 elicits a response when challenged with fatty acids of chain length of six or greater (1), although some authors have reported relative insensitivity to this chain length (11). In contrast GPR41 and GPR43 respond to C1–C6 and are inactive to longer chain lengths (2). GPR40 shares only \(~25\%\) amino acid homology to GPR43, and it is therefore probable that sequence differences between these receptors may confer the chain length specificity. There is a marked difference in the sensitivity of mGPR40 in the oocyte expression system, compared with that previously observed in the mammalian expression systems, such as HEK cells (1). This is perhaps a manifestation of the differing cellular systems employed or of the signaling pathways converging on the CACC sampled to determine that a response has occurred. Key mechanistic components additionally required for this sensitivity are perhaps absent in *Xenopus* oocytes injected with GPR40 cRNA alone; hence, the differing dose-response results from mammalian cell lines. There is also the unavoidable problem that longer-chain fatty acids, which are relatively insoluble in aqueous media, will often form micelles at higher concentrations and readily bind to plastics and glassware used in their presentation. This can greatly reduce the active unbound monomeric concentration of the fatty acid, in a manner analogous to the effects of albumin, and it remains a possibility that these problems have had more effect in the experimental system used in this study. However, short-chain fats, such as butyric acid, are water soluble, so the concentration presented is accurate.

GPR40 transcripts have been identified in the gastrointestinal tract, where the free fatty acid concentration is far higher than the circulating compartment of interest to \(\beta\)-cell physiologists, achieving levels as high as 13 mM in the small intestine and 130 mM in the colon (5). However, far lower concentrations than this have been employed in the GPR40 studies to date, indeed never reportedly >300 \(\mu\)M (11). The role of GPR40 in gastrointestinal fatty acid sensing has not been evaluated beyond a single study, suggesting that GPR40 was not responsible for unsaturated long-chain fatty acid-induced glucagon-like peptide-1 secretion in the STC-1 cell line model of intestinal L-cells, an effect ascribed to GPR120 (10). The most well-characterized enteroendocrine response to fatty acid is CCK secretion, which operates in a manner highly specific for long-chain fatty acids in STC-1 cells via mobilization of \([\text{Ca}^{2+}]_i\) (9, 15, 16).

The stimulation of GPR40 by short-chain fatty acids might suggest that several gastrointestinal physiological responses, which are specific to longer-chain-length fatty acids, cannot simply reflect a singular pathway originating from the GPR40 receptor alone. Examples of such responses include the stimulation of CCK secretion from STC-1 enteroendocrine cells (15) or the elevation of plasma CCK levels in humans (16), which are observed with medium to long-chain-length fatty acids, but not with short-chain-length fatty acids.

It is unclear whether fatty acids are acting on mGPR40 protein expressed on the plasma membrane, or operating from an intracellular location. The teleological purpose of expressing plasma membrane receptors is to allow the cell to detect and respond to signals that do not enter the cytoplasm, as is the case for most circulating hormones, cytokines, and many other factors. However, fatty acids rapidly enter all compartments of the cell (21) and the need for a purely extracellular sensor might not hold true. Indeed, receptors for endogenous lipid mediators, such as steroids and retinoids, are canonically cy-
to transplasmic and shuttle to the nucleus. The technical difficulty in addressing this experimentally is very great. In attempt to clarify this, we undertook experiments with fatty acid conjugated to CoA, which would prevent linked fatty acids entering the oocyte. Surprisingly, this approach led to the additional discovery that CoA itself stimulated the mGPR40 receptor (Fig. 5A). We were therefore unable to pursue this experimental approach to testing this hypothesis any further. In part, this would support the concept that the extracellularly facing loops of the receptor are pivotal in ligand sensing, as is classically understood for GPCRs, although CoA would not be encountered extracellularly physiologically and it might not be valid to extrapolate this interpretation to free fatty acids. However, we have previously shown that nonmetabolizable fatty acid analogues are able to stimulate CCK secretion and [Ca\(^{2+}\)], mobilization in STC-1 cells, so we do not believe it is the case that GPR40 is being activated secondary to fatty acid metabolism. It has been reported that CoA interacts with G protein-coupled receptors in an antagonistic manner, for example, by blocking the effect of ATP on the P2Y1 receptor (4). It is also interesting to reflect that an acyl chain length-dependent effect, similar to those involving CCK previously mentioned, has been reported for acyl CoA-induced Ca\(^{2+}\) release in pancreatic acinar cells (6).

Intriguingly, the CoA and fatty acid responses were both inhibited by BSA, which avidly binds fatty acids extracellularly (Fig. 5B). The possibility that the BSA is directly inhibiting the measured CaCC can be dismissed because BSA at no effect on ionomycin-stimulated currents (Fig. 5B). This agrees with the BSA inhibition of fatty acid GPR40 stimulation previously reported (11). It is intriguing to ask how fatty acid is sensed in β-cells when presented bound to proteins such as albumin, although this is not an issue for the enteroendocrine system because fatty acids are presented free or with bile salts.

In conclusion, this study has shown that mouse GPR40 heterologously expressed in Xenopus oocytes functions as a fatty acid receptor, further confirming this novel role in an additional heterologous expression system to the mammalian cell lines previously employed. The key finding was that GPR40 has the capacity to respond to fatty acids of all different chain lengths, short, medium, and long. This finding has implications for understanding the structure:function relationships of fatty acid sensors, and suggests a potential need for additional co-operative transcripts in setting cellular sensitivity and specificity to fatty acids in vivo.

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