Conserved Polar Residues in Transmembrane Domains V, VI, and VII of Free Fatty Acid Receptor 2 and Free Fatty Acid Receptor 3 Are Required for the Binding and Function of Short Chain Fatty Acids*5

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FFA2 and FFA3 are closely related G protein-coupled receptors that bind and respond to short chain fatty acids. (FFA2 and FFA3 are the provisional International Union of Pharmacology designations for the receptors previously called GPR43 and GPR41, respectively.) Sequence comparisons between these two receptors and alignments with the related G protein-coupled receptor FFA1, linked to homology modeling based on the atomic level structure of bovine rhodopsin, indicated the potential for polar residues within the transmembrane helix bundle to play important roles in ligand recognition and function. In both FFA2 and FFA3, mutation of either an arginine at the top of transmembrane domain V or a second arginine at the top of transmembrane domain VII eliminated the function of a range of short chain fatty acids. Mutation of a histidine in transmembrane domain VI, predicted to be in proximity to both the arginine residues, also eliminated function in many but not all assay formats. By contrast, mutation of a histidine in transmembrane domain IV, predicted to be lower in the binding pocket, modulated function in some assays of FFA3 function but had limited effects on the function of acetate and propionate at FFA2. Interestingly, wild type FFA3 responded to caproate, whereas FFA2 did not. Mutation of the transmembrane domain IV histidine eliminated responses of FFA3 to caproate but resulted in a gain of function of FFA2 to this six-carbon fatty acid. These data demonstrate the importance of positively charged residues in the recognition and/or function of short chain fatty acids in both FFA2 and FFA3. The development of small molecule ligands that interact selectively with these receptors will allow further details of the binding pockets to be elucidated.

Free fatty acid receptor 2 (FFA2),4 formerly known as GPR43, and free fatty acid receptor 3 (FFA3), formerly known as GPR41, are a pair of closely related, previously orphan G protein-coupled receptors (GPCRs) that respond to a range of short chain fatty acids (SCFA) (1–4). Their expression patterns in adipocytes, gastrointestinal cells, various leukocyte populations and, potentially, in pancreatic islet cells has suggested possible roles in the control of energy storage and in white cell migration and recruitment. Although variation in potencies are evident (2), particularly for acetate (C2), where FFA2 displays higher potency than FFA3, the high overlap between activating ligands for the two receptors has blurred pharmacological separation and clear resolution of their individual functions. However, this also implies that ligand recognition and binding by the two receptors is likely to share a number of similarities. Studies on other GPCRs whose ligands contain carboxylic groups indicate that positively charged amino acids within the transmembrane (TM) regions may be very important in ligand binding and/or function. For example, Tunaru et al. (5) demonstrated that an arginine residue in TM III of the high affinity nicotinic acid receptor, GPR109A (also called HM74A), provides a positive charge by which the carboxylate group of nicotinic acid can bind, whereas studies on SUCNR1 (previously known as GPR91) have shown that four arginine residues are required for binding the dicarboxylate groups of succinate (6). Basic residues have also been implicated in the binding of prostanoioids to prostanooid receptors (7) and of leukotriene B4 to the BLT1 receptor (8). Based on such observations, we have mutated basic residues that are conserved in the TM domains of human FFA2 and FFA3 to examine their contribution to the function of SCFAs. These residues are also conserved in the related GPCR, FFA1 (previously known as GPR40), which responds to medium and long chain fatty acids but not to SCFAs.

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4 The abbreviations used are: FFA, free fatty acid; eCFP, enhanced cyan fluorescent protein; eYFP, enhanced yellow fluorescent protein; FLIPR, fluorometric imaging plate reader; GPCR, G protein-coupled receptor; Gly466 → Asp Gly466, a chimeric G protein α subunit based on the backbone of Gαs but with the C-terminal 5 amino acids replaced by those from Gαolf and containing a Gly466 → Asp mutation; GTP → G, guanosine 5′-O-(thio)triphosphate; SCFA, short chain fatty acid; TM, transmembrane; PTx, pertussis toxin; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; HEK, human embryonic kidney.
Binding of Fatty Acids to FFA2/3

FIGURE 1. hFFA2 elevates [Ca\(^{2+}\)](i) in response to short chain fatty acids. A, mouse embryo fibroblasts lacking expression of both G\(_{\alpha_5}\) and G\(_{\alpha_i}\) were transfected with hFFA2 and G\(_{\alpha_i}\). The ability of acetate (10 mM) to elevate [Ca\(^{2+}\)](i) was then assessed in single cell imaging studies. Data represent mean ± S.E. from 20 individual cells. B, HEK293 cells grown on coverslips and transfected to express wild type hFFA2 (inverted triangles), hFFA2-eCFP (diamonds), hFFA2-eYFP (squares), or hFFA2-G\(_{\alpha_i}\) (crosses). Cells were loaded with Fura-2 and the effect of 10 mM acetate on [Ca\(^{2+}\)](i) was measured over time. Data represent mean ± S.E. from at least 15 individual cells expressing each construct. C, HEK293 cells grown on coverslips and transfected to express hFFA2-eCFP were loaded with Fura-2. The capacity of acetate (circles), propionate (inverted triangles), butyrate (squares), each at 10 mM, and laurate (crosses) at 100 \(\mu\)M to alter [Ca\(^{2+}\)](i), was assessed over time. Data represent mean ± S.E. from 20 individual cells selected on the basis of eCFP autofluorescence. D, HEK293 MSRII cells transiently expressing hFFA2 were exposed to varying concentrations of acetate (circles) or propionate (inverted triangles) and modulation of [Ca\(^{2+}\)](i), measured using a FLIPR-based system. Mean ± S.E., n = 3.

TABLE 1

| Short chain free fatty acid | \(pEC_{50}\) |
|-----------------------------|------------|
| Formate (C1)                | 2.8 ± 1.0  |
| Acetate (C2)                | 4.6 ± 0.2  |
| Propionate (C3)             | 4.9 ± 0.2  |
| Butyrate (C4)               | 4.4 ± 0.5  |

EXPERIMENTAL PROCEDURES

Molecular Constructs—Primers to remove the stop codon of human (h) FFA2 or hFFA3 and to introduce either a FLAG or c-myc epitope sequence were used to generate C-terminal tagged constructs in pcDNA3 (all primers and introduced restriction enzyme sites for cloning are included in supplementary data). For constructs bearing enhanced cyan or yellow fluorescent proteins (eCFP and eYFP, respectively), stop codons were removed by PCR and the amplified fragments digested and ligated into pcDNA3, in-frame, with eCFP or eYFP ligated between NotI and Xhol. Primers to amplify a pertussis toxin-insensitive variant (Cys\(^{351}\) → Ile) of G\(_{\alpha_i}\) (9) were also designed to enable ligation of hFFA3 lacking a stop codon (as above) and G\(_{\alpha_i}\) in-frame into pcDNA3.

Site-directed Mutagenesis of FFA2 and FFA3—Using the Stratagene QuikChange method, primers were used to introduce mutations into hFFA2-eYFP or hFFA3-eYFP in either pcDNA3 or pcDNA5/FRT/TO. Template was digested with DpnI to leave mutated plasmid and sequencing was carried out to confirm the introduction of the mutations.

Cell Culture and Generation of Stable Flp-In TREx HEK293 Cells—Flp-In TREx HEK293 cells (10, 11) were maintained in Dulbecco’s modification of Eagle’s medium without sodium pyruvate, with 4500 mg/liter glucose and 2 mM L-glutamine, supplemented with 10% (v/v) fetal calf serum, 1% antibiotic mixture, and 10 \(\mu\)g/ml blasticidin at 37 °C in a humidified atmosphere of air/CO\(_2\) (19:1). HEK293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% newborn calf serum and 2 mM L-glutamine and HEK293 MSRII cells were maintained in minimal essential medium containing 2 mM L-glutamine and HEK293 MSRII cells transiently expressing hFFA2 were exposed to varying concentrations of acetate (circles) or propionate (inverted triangles) and modulation of [Ca\(^{2+}\)](i), measured using a FLIPR-based system. Mean ± S.E., n = 3.

\[^{35}\text{S}]\text{GTPyS Binding Assays—[^{35}\text{S}]GTPyS binding experiments were performed using to two separate methods. For HEK293T cells transiently expressing FFA3-G\(_{\alpha_3}\),[^{35}\text{S}]GTPyS binding was initiated by the addition of 2.5 \(\mu\)g of cell membranes and assessed by immunoprecipitation with a G\(_{\alpha_3}\)-selective antiserum (12), according to the method described in Stoddart et al. (13).[^{35}\text{S}]GTPyS incorporation was also assessed using 15 \(\mu\)g of cell membranes from stable Flp-In TREx HEK293 cells expressing FFA3-eYFP and terminated by rapid filtration with a Brandel cell harvester (14). Single Cell Calcium Assays—HEK293 cells and EF88 cells (mouse embryo fibroblasts derived from G\(_{\alpha_5}\) + G\(_{\alpha_i}\) knockout mouse lines) (15) grown on poly-D-lysine-coated coverslips were transiently transfected to express the construct of interest. For Flp-In TREx HEK293 cells these were treated with or without 1 \(\mu\)g/ml doxycycline. 24 h after transfection or cell treatment the cells were loaded with the calcium-sensitive dye,
**SCFAs activate hFFA3: [35S]GTPγS binding and [Ca^{2+}]<sup>2</sup> mobilization studies.** A, HEK293T cells were transfected with the hFFA3-Cys<sup>351→Ile</sup> Gα<sub>i3</sub> fusion protein (20). Membranes were prepared from these cells and employed in [35S]GTPγS binding studies performed in the presence of varying concentrations of individual SCFAs ranging from C2 (acetate) to C6 (caproate). Cys<sup>351</sup> was recovered at the end of experiment by immunoprecipitation with a selective antiserum (12). Data points represent mean ± S.E. from three experiments.

**Calcium Assays Using Cell Populations—**These were performed using Flp-In TREP HEK293 cells harboring hFFA2-eYFP that were treated with or without 1 μM doxycycline. Cells were grown in poly-d-lysine-coated wells of a 96-well microtiter plate. 24 h after transfection, cells were loaded with the calcium-sensitive dye Fura-2, as above, and the effect of potential ligands was assessed using a FLEXStation (Molecular Devices).

**FLIPR-based Calcium Assay—**HEK293 MSRII cells were transiently transfected with either wild type or mutant FFA2-eYFP or FFA3-eYFP and seeded at 15,000 cells/well into poly-d-lysine-coated 384-well black-wall, clear-bottom microtiter plates (Greiner Bio-One, Kremsmünster, Austria) using a Multidrop dispenser. The following day, cells were washed once and placed in 40 μl of assay buffer (Hanks’ balanced salts solution, 10 mM HEPES, 200 μM Ca<sup>2+</sup>, 2.5 mM probenicid, 0.5 mM brilliant black, 1 μM fluo-4 AM) for 1 h at 37 °C before assay with a fluorometric imaging plate reader (FLIPR, Molecular Devices, Sunnyvale, CA). Images were collected every 2 s and agonist was added after 10 s for a total of 60 images.

**Cell Lysate Generation and Phospho-ERK1/2 Assays—**FFA2 or FFA3 stable Flp-In TREP HEK293 cells were seeded onto 12-well poly-d-lysine-coated plates and induced with 1 μg/ml doxycycline for 48 h. Cells were serum starved overnight before assay and, in the case of pertussis toxin (PTx) treatment, incubated with 25 ng/ml PTx. After stimulation, cells were har-

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**TABLE 2**

**Potencies of short chain free fatty acids as agonists at hFFA3**

| Short chain free fatty acid | pEC<sub>50</sub> |
|-----------------------------|-----------------|
| Acetate (C2)                | 1.8 ± 0.7       |
| Propionate (C3)             | 3.6 ± 0.1       |
| Butyrate (C4)               | 3.6 ± 0.2       |
| Valerate (C5)               | 3.9 ± 0.2       |
| Caproate (C6)               | 3.2 ± 0.1       |

Fura-2; 1.5 μM Fura-2 was added to normal growth media and the cells incubated at 37 °C for 30 min. Coverslips were then placed into a microscope chamber containing physiological saline solution and illuminated with an ultra high point intensity 75-watt xenon arc lamp (Optosource, Cairn Research, Faversham, Kent, UK) and imaged using a Nikon Diaphot inverted microscope equipped with a Nikon ×40 oil immersion Fluor objective lens (NA = 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 CCD camera (Cool Snap-HQ, Roper Scientific/Photometrics, Tucson, AZ). MetaFluor imaging software (Universal Imaging Corp., Downing, PA) was used for control of the monochromator, CCD camera, and for processing of the cell image data. Sequential images (2 × 2 binning) were collected every 2 s, 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—**These were performed using Flp-In TREP HEK293 cells harboring hFFA2-eYFP that were treated with or without 1 μg/ml doxycycline. Cells were grown in poly-d-lysine-coated wells of a 96-well microtiter plate. 24 h after transfection, cells were loaded with the calcium-sensitive dye Fura-2, as above, and the effect of potential ligands was assessed using a FLEXStation (Molecular Devices).

**FLIPR-based Calcium Assay—**HEK293 MSRII cells were transiently transfected with either wild type or mutant FFA2-eYFP or FFA3-eYFP and seeded at 15,000 cells/well into poly-d-lysine-coated 384-well black-wall, clear-bottom microtiter plates (Greiner Bio-One, Kremsmünster, Austria) using a Multidrop dispenser. The following day, cells were washed once and placed in 40 μl of assay buffer (Hanks’ balanced salts solution, 10 mM HEPES, 200 μM Ca<sup>2+</sup>, 2.5 mM probenicid, 0.5 mM brilliant black, 1 μM fluo-4 AM) for 1 h at 37 °C before assay with a fluorometric imaging plate reader (FLIPR, Molecular Devices, Sunnyvale, CA). Images were collected every 2 s and agonist was added after 10 s for a total of 60 images.

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vested in ice-cold radioimmunoprecipitation assay buffer (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, and 0.5% sodium deoxycholate supplemented with 10 mM NaF, 5 mM EDTA, 10 mM NaH₂PO₄, 5% ethylene glycol, 1 mM Na₃VO₄ and a protease inhibitor mixture (Complete; Roche Diagnostics), pH 7.4) and the supernatant recovered after centrifugation at 4 °C for 15 min at 14,000 × g. Active (phospho) and total extracellular signal-regulated mitogen-activated protein kinases 1/2 (ERK1/2) were detected by Western immunoblotting. Quantitation was achieved by densitometric analysis.

Homology Modeling—Homology models of hFFA2 and hFFA3 were generated based on the atomic level structure of bovine rhodopsin using the automated GPCR_Builder program. The process of developing the model is described in detail by Berkhour et al. (16). The intracellular loops of the receptors were not modeled due to their poor definition in the crystal structures of rhodopsin. The receptors were aligned based on key conserved residues in class A GPCRs, these include an Asn in TM I, a Cys residue in extracellular loop 1, the D(E)RY motif at the bottom of TM III, a Cys in extracellular loop 2, and Pro residues in TM V and TM VI. FFA2 contains an ERY motif at the base of TM III, whereas in FFA3 it is an ERF motif.

Data Analysis—All data were quantified and analyzed using GraphPad Prism 4.0. Differences were considered statistically significant when \( p < 0.05 \) according to one-way analysis of variance. Multiple analyses of the significance of differences in ERK MAP kinase phosphorylation studies were compared via one-way analysis of variance with Bonferroni's multiple comparisons of the mean post hoc test.

RESULTS

FFA2 has been reported to interact with Goq family G proteins (17–19) and to be activated by SCFAs. To confirm this, hFFA2 was ex-

FIGURE 3. Alignments and homology models of FFA2 and FFA3. A, amino acid sequences corresponding to hFFA2, hFFA3, and hFFA1 were aligned using the ClustalX algorithm. Residues are colored as follows; small and hydrophobic residues are colored red, acidic residues colored blue, basic residues colored magenta, hydroxyl and amine containing residues are colored green. Predicted transmembrane elements are boxed and conserved basic residues located either within the transmembrane helices or at the interface of a transmembrane helix and the extracellular space are highlighted. Accession numbers are as follows: hFFA1, NP005294; hFFA2, NP005297; and hFFA3, NP005295. B, homology models of the transmembrane elements of hFFA2 (left-hand side) and hFFA3 (right-hand side) were generated using GPCR_Builder based on the atomic level structure of bovine rhodopsin. The conserved basic residues identified in A are specified. Individual TM helices are color coded. Upper panels show space filling models of the receptors viewed from the extracellular medium. Lower panels explore predicted positions of the amino acids that may contribute to the binding of SCFAs that are highlighted in the upper panels, viewed from the side.
pressed transiently along with Goqi in mouse embryo fibroblasts (EF88 cells) (15) that lack expression of both Goqi and Goq11. In single cell Ca2+-imaging studies a large, transient rise in [Ca2+]i was recorded in response to the addition of the C2 SCFA, acetate (Fig. 1A). Because EF88 cells are difficult to transfect with high efficiency, subsequent studies were performed using HEK293T cells that endogenously express both Goqi and Goq11.

Following introduction of hFFA2, 10 mM acetate also caused a transient and data not shown) using hFFA2-eCFP, eYFP, or Goq transient in response to propionate when the receptor was introduced into HEK293T cells, suggesting that it is unable to couple to authentic Goqi family G proteins (Fig. 2C, co-expression of hFFA3 with the chimeric G protein Gly66 → Asp Goqi11 (22, 23) resulted in a rapid and transient increase in [Ca2+]i in response to propionate (Fig. 2C).

Sequence alignment of hFFA2 and hFFA3, along with hFFA1 (24, 25), demonstrated that there are a number of conserved basic residues in TM regions of these GPCRs: an arginine or lysine in TM II (2.60 in the numbering system), histidines in TM IV (4.56) and TM VI (6.55), an arginine in TM V (5.39), an aspartate in TM VII (7.35), a glycine in TM III (T.53), and a further arginine at the top of TM VII (7.35) (Fig. 3A). These were hence candidates to act as charge partner(s) for the carboxylate function of the SCFAs. Homology models (16) of the transmembrane regions of hFFA2 and hFFA3 were generated (Fig. 3B) based on the atomic level structure of bovine rhodopsin. The size and low potency of the SCFAs prevented them from being docked with any degree of accuracy into the models of hFFA2 and hFFA3. However, the models of both receptors predict that Arg(5.39) (Arg180 in 18), including Goq11 (17). Such Gt-family G proteins are well suited to the use of [35S]GTPyS binding studies to examine G protein activation by GPCRs. Both with transient co-transfection of hFFA3 and Goq11 into HEK293T cells (3) and following transfection of a hFFA3-Goq11 fusion protein, propionate increased levels of Goq11-bound [35S]GTPyS in membranes of these cells in a concentration-dependent manner (Fig. 2A) and with similar potency. The hFFA3-Goq11 fusion protein and [35S]GTPyS binding assay demonstrated similar potencies for propionate, butyrate, and valerate (C5) at hFFA3 but a distinctly lower potency for acetate (Fig. 2A and Table 2), in accord with other studies (17, 18). We have previously shown that addition of bovine serum albumin results in binding of long chain fatty acids and, therefore, restricts long chain fatty acid-mediated activation of hFFA1 (13). By contrast, addition of bovine serum albumin along with Goqi in mouse embryo fibroblasts (EF88 cells) (15) that lack expression of both Goqi and Goq11, in single cell Ca2+-imaging studies a large, transient rise in [Ca2+]i, was recorded in response to the addition of the C2 SCFA, acetate (Fig. 1A). Because EF88 cells are difficult to transfect with high efficiency, subsequent studies were performed using HEK293T cells that endogenously express both Goqi and Goq11. Following introduction of hFFA2, 10 mM acetate also caused elevation of [Ca2+]i in these cells (Fig. 1B). This was also the case when C-terminal-modified forms of hFFA2 were employed that incorporated either the c-myc or FLAG epitope tag sequences or generated hFFA2 fusion proteins with each of eCFP, eYFP, or Goqi (Fig. 1B and data not shown). Using hFFA2-eCFP as an exemplar, similar responses in terms of both amplitude and kinetics of the signal were produced in transiently transfected HEK293T cells upon addition of each of acetate, propionate (C3), and butyrate (C4) but not the longer chain, saturated fatty acid laurate (C12) (Fig. 1C) that is an agonist at the related receptor FFA1 (13). Single cell Ca2+-imaging studies are ideal to examine kinetics of the Ca2++ transient in response to receptor activation. However, they are poorly suited to generate information on ligand potency. To do so we employed fluorometric imaging plate reader (FLIPR)-based [Ca2+]i assays. These demonstrated that the potency of acetate, propionate, and butyrate was not substantially different following transient expression of hFFA2-eYFP in HEK293 cells (Fig. 1D and Table 1), whereas formate (C1), although displaying agonism, was substantially less potent (Table 1).

Although FFA2 and FFA3 respond to a similar range of SCFAs (17, 18), in contrast to FFA2, FFA3 is reported to be coupled selectively to PTx-sensitive, Gi family G proteins (17, 18), including Goq11 (17). Such Gt-family G proteins are well suited to the use of [35S]GTPyS binding studies to examine G protein activation by GPCRs. Both with transient co-transfection of hFFA3 and Goq11 into HEK293T cells (3) and following transfection of a hFFA3-Goq11 fusion protein, propionate increased levels of Goq11-bound [35S]GTPyS in membranes of these cells in a concentration-dependent manner (Fig. 2A) and with similar potency. The hFFA3-Goq11 fusion protein and [35S]GTPyS binding assay demonstrated similar potencies for propionate, butyrate, and valerate (C5) at hFFA3 but a distinctly lower potency for acetate (Fig. 2A and Table 2), in accord with other studies (17, 18). We have previously shown that addition of bovine serum albumin results in binding of long chain fatty acids and, therefore, restricts long chain fatty acid-mediated activation of hFFA1 (13). By contrast, addition of bovine serum albumin along with Goqi in mouse embryo fibroblasts (EF88 cells) (15) that lack expression of both Goqi and Goq11, in single cell Ca2+-imaging studies a large, transient rise in [Ca2+]i, was recorded in response to the addition of the C2 SCFA, acetate (Fig. 1A). Because EF88 cells are difficult to transfect with high efficiency, subsequent studies were performed using HEK293T cells that endogenously express both Goqi and Goq11.

It is important to note that the ability of acetate (A and C) or propionate (B and D) to elevate [Ca2+]i, was then assessed either in single cell imaging studies (A and B) (mean ± S.E. from at least 20 individual cells) or in cell population studies using a FLIPR (C and D) (mean ± S.E., n = 3).

FIGURE 4. Mutation of arginine 5.39 in hFFA2 eliminates the function of short chain fatty acids. A, hFFA2-eYFP (red), Arg180 → Ala (5.39) hFFA2-eYFP (magenta), Arg180 → Lys(5.39) hFFA2-eYFP (green), Arg180 → Leu(5.39) hFFA2-eYFP (blue), or Arg180 → Ser(5.39) hFFA2-eYFP (cyan) were expressed transiently in HEK293 cells. The ability of acetate (A and C) or propionate (B and D) to elevate [Ca2+]i, was then assessed either in single cell imaging studies (A and B) (mean ± S.E. from at least 20 individual cells) or in cell population studies using a FLIPR (C and D) (mean ± S.E., n = 3).
hFFA2 and Arg^{185} in hFFA3) points into the core of the receptor, with His(4.56) (His^{140} in hFFA2 and His^{146} in hFFA3) in proximity, although this His is predicted to be more shielded from Arg(5.39) in hFFA3 (Fig. 3B). By contrast, although located close to the extracellular environment, in these models, the Arg/Lys in TM II pointed away from the water-filled cavity within the TM domains, which would make it unavailable for interaction with the carboxylate group of a SCFA binding within this cavity. Arg(7.35) (Arg^{255} in hFFA2 and Arg^{258} in hFFA3) located at the top of TM VII in FFA1 has recently been implicated in interactions with the carboxylate group of a medium chain fatty acid (27, 28), whereas His(6.55) conserved between hFFA2 and hFFA3 at the top of TM VI (His^{242} in hFFA2 and His^{245} in hFFA3) was predicted to be in close proximity to Arg(7.35) (Fig. 3B).

To explore the importance of these amino acids for agonist recognition and/or function we initially mutated Arg(5.39) in the context of hFFA2-eYFP to a range of other amino acids including Ala, Lys, Leu, and Ser. Transient introduction of each of these mutants into HEK293 cells failed to produce elevation of [Ca^{2+}], in response to either acetate or propionate whether studies were performed via single cell calcium imaging (Fig. 4, A and B) or FLIPR-based, cell population (Fig. 4, C and D) assays. Despite employing hFFA2-eYFP in these studies to allow visualization of the expression and cellular distribution of wild type and Arg^{180} → Xaa mutants of hFFA2, image resolution was not of sufficient quality to ensure that the loss of agonist function in the mutants was not simply associated with poor cell surface delivery. To explore this we generated Flp-In TREx HEK293 cells in which either wild type hFFA2-eYFP or Arg^{180} → Ala(5.39) hFFA2-eYFP was cloned into the Flp-In TREx locus. Such cells allow tetracycline/doxycycline-induced turn-on of expression of DNAs cloned into the Flp-In site (10, 29, 30). In parallel, and based on the molecular models described above, we also generated equivalent cells to allow regulated expression of His^{140} → Ala(4.56) hFFA2-eYFP, His^{242} → Ala(6.55) hFFA2-eYFP, and Arg^{255} → Ala(7.35) hFFA2-eYFP (Fig. 5A). In each case the construct was undetectable in the absence of doxycycline (Fig. 5A) but following treatment with doxycycline (1 μg/ml, 24 h), each of the constructs was expressed and cell imaging indicated a
large proportion of each construct to be located at the cell surface (Fig. 5A). Anti-fluorescent protein immunoblot studies performed on SDS-PAGE resolved lysates of these cells confirmed turn-on of expression of all forms of hFFA2-eYFP in response to doxycycline (data not shown). The capacity of the cells to respond to SCFAs was then explored. Cell population [Ca^{2+}]_{i} studies demonstrated that both acetate and propionate elevated [Ca^{2+}]_{i}, in a concentration-dependent fashion via wild type hFFA2 and confirmed the lack of response of Arg^{180} \rightarrow Ala{5(39)} hFFA2-eYFP to either of these ligands (Fig. 5B and Table 3). Similarly, Arg^{255} \rightarrow Ala{7(35)} hFFA2-eYFP and His^{242} \rightarrow Ala{6(55)} hFFA2-eYFP failed to respond to either acetate or propionate (Fig. 5B). By contrast, His^{140} \rightarrow Ala{4(56)} hFFA2-eYFP did respond to both acetate and propionate, however, the observed potency of each ligand was reduced compared with the wild type receptor (Fig. 5B).

In recent times, it has become clear that GPCRs can adopt different conformations that may regulate distinct G proteins and downstream effectors in a non-equivalent manner and can therefore result in either distinct rank orders of potency or altered pharmacological responses (31, 32). To explore the pathways of activation of the wild type hFFA3 receptor construct, propionate increased binding of [35S]GTPyS in a concentration-dependent fashion with pEC_{50} = 4.00 \pm 0.06. In contrast, none of the mutants responded to propionate (Fig. 6B). As noted earlier, hFFA3 can generate Ca^{2+} signals in response to propionate when an appropriate chimeric G protein is present. Transient introduction of either Go_{q} or Gly^{66} \rightarrow Asp Go_{q15} into Flp-In TREx HEK293 cells harboring hFFA3-eYFP at the Flp-In locus resulted in equivalent and very small Ca^{2+} transients upon addition of propionate (Fig. 6C). Without introduction of Gly^{66} \rightarrow Asp Go_{q15} the response to propionate was not increased when hFFA3-eYFP expression was induced (Fig. 6C). However, with co-expression of Gly^{66} \rightarrow Asp Go_{q15} and induction of hFFA3-eYFP, propionate was able to generate a robust and more sustained elevation of [Ca^{2+}]_{i}, (Fig. 6C). Single cell [Ca^{2+}]_{i} imaging studies performed on cells induced to express either hFFA3-eYFP or one of the hFFA3 mutants and transfected to transiently express Gly^{66} \rightarrow Asp Go_{q15} resulted in elevation of [Ca^{2+}]_{i}, in response to propionate via His^{146} \rightarrow Ala{4(56)} hFFA3-eYFP as well as wild type hFFA3-eYFP but not via the other mutants (Fig. 6C).

Because of the differences in response of His^{146} \rightarrow Ala{4(56)} hFFA3-eYFP to propionate in the Ca^{2+} imaging and [35S]GTPyS binding assays, we also explored the ability of these cells to respond to acetate and propionate in ERK1/2 phosphorylation assays (Fig. 6D). Induction of hFFA3-eYFP expression did not enhance basal ERK1/2 phosphorylation but acetate, and more effectively propionate, increased phosphorylation of ERK1/2 via hFFA3 (Fig. 6D). As with hFFA2, Arg^{185} \rightarrow Ala{5(39)} hFFA3-eYFP, His^{245} \rightarrow Ala{6(55)} hFFA3-eYFP, and Arg^{258} \rightarrow Ala{7(35)} hFFA3-eYFP failed to respond to either acetate or propionate in these assays (Fig. 6D). His^{146} \rightarrow Ala{4(56)} hFFA3-eYFP produced a moderate enhancement of ERK1/2 phosphorylation in response to propionate but not acetate (Fig. 6D). Pre-treatment of cells induced to express hFFA3-eYFP with PTx virtually eliminated the response to both acetate and propionate (Fig. 6D), whereas pre-treatment with YM-254890 was without effect (Fig. 6D), consistent with the predominant coupling of hFFA3 to Go family G proteins.

The loss of agonist action of acetate and propionate at Arg^{5(39)} \rightarrow Ala and Arg^{7(35)} \rightarrow Ala hFFA2 and hFFA3 suggested that it might be possible to generate mutants that dis-
were treated with doxycycline to induce the receptor construct. The ability of acetate or propionate to induce hFFA2-eYFP or the individual point mutants described in comparisons with the agonist effect in the absence of treatment,‡ is substantial signal when the cells were co-transfected to express Gly 66 Arg 258 5.39 or arginine 7.35 eliminates function. histidine 4.56 alters function in an end point-dependent fashion, whereas mutation of arginine positions. As such, we generated Arg180 Glu hFFA2-eYFP and Arg255 Glu hFFA2-eYFP as well as Arg185 Glu hFFA3-eYFP and Arg258 Glu hFFA3-eYFP. Each of these was again used to generate inducible Flp-In TREx HEK293 cells harboring one of these mutants (Figs. 7 and 8). However, although induced expression of each of the mutants was achieved (Figs. 7A and 8A) and each, as anticipated from the foregoing, lost the ability to respond to acetate and propionate in all of the assay end points tested in no case was there gain of function to acetamide or propionamide (Figs. 7, B and C, and 8, B–E). A marked gain of function was, however, noted for His140 → Ala(4.56) hFFA2-eYFP. Although wild type hFFA2-eYFP did not respond to the SCFAs caproate (C6) and caprylate (C8) in Ca2+ mobilization assays, both of these ligands induced concentration-dependent elevation of [Ca2+]i, in cells expressing His140 → Ala(4.56) hFFA2-eYFP (Fig. 9, A and B). In ERK1/2 phosphorylation assays, His140 → Ala(4.56) hFFA2-eYFP also responded to caproate but not significantly to caprylate (Fig. 9C). Interestingly, hFFA3-eYFP expressing cells generated a substantial ERK1/2 MAP kinase response to caproate and a weaker response to caprylate (Fig. 9C). However, His146 → Ala(4.56) hFFA3-eYFP failed to respond significantly to any ligand tested. The agonist action of caproate at hFFA3-eYFP was verified in [35S]GTPγS binding studies performed on membranes derived from cells induced to express this construct (Fig. 9D). Again, in this assay His146 → Ala(4.56) hFFA3-eYFP did not respond to any ligand tested (Fig. 9D), including a series of medium and longer chain free fatty acids known to activate FFA1 (data not shown).

DISCUSSION

FFA2 and FFA3 are receptors for SCFAs (17–19). They have attracted considerable attention as prospective therapeutic targets for the treatment of conditions ranging from inflammation to obesity, based on their distribution pattern in leukocytes, the ability of propionate to increase produc-
Binding of Fatty Acids to FFA2/3

FIGURE 7. Charge reversal mutations of arginine 5.39 and arginine 7.35 in hFFA2 fail to induce responses to acetamide. A, Flp-In TREx HEK293 cells harboring either Arg180→Glu(5.39) hFFA2-eYFP or Arg255→Glu(7.35) hFFA2-eYFP at the Flp-In locus were untreated (left-hand panel) or treated with doxycycline (1 μg/ml, 24h) (right-hand panel). eYFP autofluorescence was then visualized. B and C, the capacity of acetate (C2), propionate (C3), acetamide (AM), or propionamide (PM) (each at 10 mM) to elevate intracellular Ca2+ (B) (mean ± S.E. n = 3, **p < 0.001 compared with unstimulated) or induce phosphorylation of the ERK1/2 MAP kinases (C) (a representative example of two experiments is shown) in cells expressing hFFA2-eYFP or Arg180→Glu(5.39) hFFA2-eYFP was assessed as described under “Experimental Procedures.” In the ERK1/2 MAP kinase studies fetal bovine serum (FBS) acted as a positive control.

fatty acids, and small molecule agonists have been identified and developed (34–38). This reflects, in part, that FFA1 is highly expressed in pancreatic islets, that ligands at this receptor modulate glucose-dependent insulin secretion and that FFA1 is, therefore, considered an interesting potential target for the treatment of type 2 diabetes (20, 35, 38, 39). The availability of such ligands assisted greatly in the validation of the ligand binding pocket of FFA1 that had been based initially on a mutational strategy (27). We constructed homology models of the TM domains of hFFA2 and hFFA3 and took advantage of the relatedness of these receptors to FFA1 in considering amino acids likely to contribute to ligand binding and/or function of SCFAs in hFFA2 and hFFA3. Based on studies of FFA1 (27, 28) and other GPCRs that respond to ligands containing one or more key carboxylate groups (5, 6), the carboxylate moiety of the SCFAs is likely to be coordinated by one or more basic residues in, or at the top, of TM domains of these receptors. These are expected to face into the water-filled cavity generated by the organization of the seven TM helices. Sequence alignment of hFFA1 with hFFA2 and hFFA3 indicated that arginine residues at 5.39 and 7.35 met these selection criteria, whereas an arginine (hFFA3) or a lysine (hFFA2) at the top of TM domain II did not. Furthermore, a histidine in TM domain VI (6.55) was predicted to be in apposition to both arginine 5.39 and 7.35 and the asparagine at 6.55 in FFA1 has been shown to play an important role in ligand binding or function (27). A histidine in TM domain IV (4.56) of both hFFA2 and hFFA3 was also considered of interest because it is conserved in FFA1. Because it is predicted to sit lower in the TM domains than the 6.55 histidine, we considered that it might play a role in fatty acid chain length selectivity.

Initially, we confirmed the previously described basic pharmacology and G protein selectivity of signaling of FFA2 and FFA3 via a series of transient transfection studies. In mutagenesis studies it is challenge to ensure that loss of function is not simply a reflection of important roles of the amino acids selected for modification in protein folding and/or cell surface delivery. Indeed, following initial studies on point mutants of hFFA2 linked to eYFP to allow visualization it was not possible for us to be certain if the lack of response of the Arg(5.39) mutants of hFFA2 to SCFAs was truly a reflection of the importance of this amino acid or due to poor targeting of the mutant to the cell surface. This reflected that imaging of the transfected cells indicated a substantial proportion of each receptor variant, including the wild type, was retained intracellularly (not shown). However, these initial studies appeared to indicate that arginine 5.39 was vital for function and could not be replaced, even by lysine. To provide comfort on this issue we, therefore, initiated a program to generate stable cell lines expressing eYFP-tagged forms of each of wild type hFFA2 and hFFA3 and Xaa to Ala mutants of each receptor at amino acids 4.56, 5.39, 6.55, and 7.35. Each of these mutants was delivered effectively to the surface of cells. Furthermore, we chose to generate cell lines able to express the proteins in an inducible fashion to eliminate potential effects of SCFAs that were not mediated via the introduced constructs because fatty acids in
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FIGURE 8. Charge reversal mutations of arginine 5.39 and arginine 7.35 in FFA3 also fail to induce responses to acetamide. A, Flp-In TREX HEK293 cells harboring either Arg358 → Glu(5.39) hFFA3-eYFP or Arg358 → Glu(7.35) hFFA3-eYFP at the Flp-In locus were untreated (left-hand panel) or treated with doxycycline (1 μg/ml, 24 h) (right-hand panel). eYFP autofluorescence was then visualized. B and C, the capacity of acetate (C2), propionate (C3), or acetamide (AM) (each at 10 mM) to increase binding of [35S]GTPγS (B) (mean ± S.E. n = 3, * p < 0.01 compared with unstimulated) or induce phosphorylation of the ERK1/2 MAP kinases (C) (a representative example of two experiments is shown) in cells expressing hFFA3-eYFP, Arg358 → Glu(5.39) hFFA3-eYFP, Arg358 → Glu(7.35) hFFA3-eYFP was assessed as described under “Experimental Procedures.” D and E, equivalent studies explored the effects of acetate (D) and acetamide (E) to elevate intracellular Ca2+ in individual cells induced to express hFFA3-eYFP, Arg358 → Glu(5.39) hFFA3-eYFP, or Arg358 → Glu(7.35) hFFA3-eYFP and that were transfected to express Gly66 → Asp Gαs transiently.

general are known to generate a variety of effects in various cells and tissues that are independent of cell surface receptors. To do so we employed the Flp-In TREX system in HEK293 cells as we have found tetracycline/doxycycline control of construct expression to be tightly regulated (10, 11, 14, 29, 30). Studies on the hFFA2 receptor expressing cell lines initially employed measures of the elevation of Ca2+ based on the known preference of FFA2 to activate Gq/G11 G proteins and the stable expression system allowed easy assessment of ligand potency at the various mutants. In accordance with the function in FFA1, mutation of either Arg(5.39) or Arg(7.35) entirely eliminated responses to SCFAs. Furthermore, so did mutation of His(6.55). Mutation of His(4.56) reduced the potency of SCFAs to elevate [Ca2+]i, although at very high concentrations these ligands were able to produce a close to maximal effect. These results, linked to related previous studies on FFA1 (27), indicate that amino acids at the top of TM segments V, VI, and VII of hFFA2 are likely important for coordinating the binding and function of SCFAs, with a modulatory role for His(4.56). However, in the absence of high affinity ligands of FFA2 that can be used in binding studies, it is not possible to discriminate between effects of the mutations on the recognition and binding of the SCFAs and potentially distinct effects on signal transduction. It is clear that a number of pharmaceutical companies have active programs to discover high affinity, small molecule regulators of these receptors. However, at this point no useful information is available in the public domain. When such ligands become available the set of mutants we have generated will be very useful to characterize the contributions of each amino acid to ligand recognition versus signal generation.

FFA3 also responds to SCFAs and, based on the results from hFFA2, it was not surprising that mutation of Arg(5.39), Arg(7.35), and His(6.55) in hFFA3 eliminated function of the SCFAs exemplified by propionate. Importantly, however, not all mutations of charged amino acids in hFFA3 result in loss of function. Mutation of either of two Arg residues in the 1st intracellular loop of FFA3 to the corresponding amino acids in the closely related (but non-functional), prospective receptor GPR42 was without effect on the function of SCFAs (17). Despite the overlap of the ligands that activate FFA2 and FFA3, the structure-activity relationship of SCFAs at hFFA3 is separable from hFFA2 and this suggests that it may be possible to identify small molecule ligands that will be selective between these two GPCRs. Thus, it was of interest that in studies on the capacity of hFFA3 to promote [35S]GTPγS binding of His(4.56) also eliminated function. The structural basis of this is unclear from the molecular models. However, it has become obvious in recent times that the details of ligand pharmacology and function of many GPCRs can vary dependent upon the end point measured. This is generally considered to reflect the capacity of different ligands to stabilize or enrich different conformations of the receptor (31, 32). As G proteins are allostery regulators of the binding of agonist ligands to GPCRs, it would not be surprising if the detailed agonist pharmacology of a receptor was therefore distinct for signals transduced via different G proteins. We assessed this for the hFFA3 mutants by comparing their ability to promote binding of [35S]GTPγS binding to PTx-sensitive G proteins and to elevate [Ca2+]i, via a promiscuous, chimeric G protein. Evidence in favor of this model was that His(4.56) → Ala hFFA3 responded effectively to proprionate in the [Ca2+] assay. As a further extension we examined the capacity of acetate and propionate to promote phosphorylation of the ERK1/2 MAP kinases via wild type and mutated forms of hFFA3. As before, the Arg(5.39), Arg(7.35), and His(6.55) mutants were unable to generate signals but proprionate displayed agonism with reduced efficacy at the His(4.56) mutant. These observations encouraged us to also examine hFFA2 and the mutant forms of this GPCR in the ERK1/2 MAP kinase assay. As predicted
From the foregoing, the Arg(5.39) and Arg(7.35) mutants of FFA2 displayed no activity, the His(4.56) mutant was functional and now the His(6.55) mutant could be shown to retain some degree of function, although to propionate but not to acetate. As a further indication of the variations in pharmacology of FFA2 and FFA3, hFFA3 was able to both promote enhanced ERK1/2 MAP kinase phosphorylation and[^35S]GTPγS binding in response to caproate (C6) but not to caprylate (C8). Mutation of His(4.56) in hFFA3 eliminated this activity in both assays. By contrast, hFFA2 did not respond to either of these ligands in either ERK1/2 MAP kinase or [Ca^{2+}] mobilization assays. However, mutation of His(4.56) in hFFA2 resulted in a gain of function of this receptor to caproate and caprylate in [Ca^{2+}] assays and to caproate and, very weakly, to caprylate in ERK1/2 MAP kinase assays. Such studies suggest that His(4.56) either is a residue important in chain length selectivity for SCFAs between hFFA2 and hFFA3 or is at least located in a position that influences this pharmacology. Compared with similar mutations in FFA1, where a number of the alterations tended to produce potency shifts for the agonists (27, 28), we noted that for hFFA2 and hFFA3, many of these resulted in ablation of agonist function. This may reflect that the longer chain length of fatty acids that are agonists at FFA1 provide the opportunity for a larger number of amino acids in the binding pocket to contribute to binding. The small size of ligands such as acetate and propionate defines that this is unlikely to be the case for hFFA2 and hFFA3. Again, the identification and development of small molecule ligands distinct from the SCFAs would be invaluable in efforts to define the contribution of His(4.56) to receptor function and pharmacology. The low potency of the SCFAs for both hFFA2 and hFFA3 make it impractical to differentiate between effects of the mutations studied in ligand recognition versus activation of these receptors. However, both because of the initiation of an understanding of the ligand binding site produced from these studies, and the interest of the pharmaceutical industry in these GPCRs as potential therapeutic targets, it is likely that small molecule ligands with greater affinity and potentially selectivity between FFA2 and FFA3 will be uncovered.

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