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Mutational analysis of G-protein coupled receptor – FFA2

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ABSTRACT

FFA2 (GPR43) is a receptor for short-chain fatty acids (SCFAs), acetate, and propionate. FFA2 is predominantly expressed in islets, a subset of immune cells, adipocytes, and the gastrointestinal tract which suggest a possible role in inflammatory and metabolic conditions. We have previously described the identification and characterization of novel phenylacetamides as allosteric agonists of FFA2. In the current study, we have investigated the molecular determinants contributing to receptor activation with the endogenous and synthetic ligands as well as allosteric interactions between these two sites. The mutational analysis revealed previously unidentified sites that may allosterically regulate orthosteric ligand's function as well as residues potentially important for the interactions between orthosteric and allosteric binding sites.

1. Introduction

Free fatty acid receptor 2 (FFA2), also known as GPR43, is a member of the G-protein-coupled receptor (GPCR) superfamily [1]. GPCRs are known to bind to structurally diverse ligands and many are molecular targets for a wide range of therapeutic molecules [2,3]. FFA2 forms a subfamily with two other closely related receptors, FFA1 (GPR40) and FFA3 (GPR41), which are clustered on chromosome 19q13.1 in humans and share ~30–40% sequence identity [1]. All three receptors can be activated by free fatty acids (FFA) but show distinct structure activity relationships for different chain lengths [4,5], with short-chain fatty acids (SCFAs, six or fewer carbon molecules) activating FFA2 and FFA3, and medium- and long-chain fatty acids activating FFA1 [6]. FFA2 can couple to both $G_{\alpha i}$ and $G_{\alpha q}$ [7,8] and is activated by C2 (acetate) and C3 (propionate) SCFAs [7,9].

Given the important physiological effects of FFAs, there is substantial interest in exploring this subfamily of receptors as novel targets for various diseases [5]. FFA2 expression has been reported to be enriched in islets, a subset of immune cells, adipocytes, and the gastrointestinal tract, suggesting its potential role in various inflammation and metabolic conditions [7–10]. Clearly, the identification of pharmacological tools and a better understanding of the receptor function will facilitate the development of potential therapeutic molecules targeting this receptor.

FFA1 has attracted considerable attention given its well-established effects on pancreatic β-cell function and its potential utility in diabetes treatment [11,12]. Molecular modeling and mutagenesis studies have identified several charged residues located in transmembrane domains (TM) 5, 6, and 7 that are important for anchoring carboxylate groups in long-chain fatty acids [13,14]. These polar residues are also conserved in FFA2 and FFA3, and similar to FFA1, they have been shown to be important for FFA2 and FFA3 endogenous ligand binding and function [15,16].

We have recently described the identification and characterization of a series of phenylacetamide derivatives as FFA2 allosteric agonists [17,18]. We herein report the results of mutagenesis studies on the receptor, and the identification of previous unknown residues that may affect receptor activation as well as residues important for allosteric interactions on FFA2.

2. Material and methods

2.1. Materials

Reagents were purchased from various sources as follows: sodium acetate from Sigma (St. Louis, MO), propionate from Fluka Chemie (Taufkirchen, Germany), coelenterazine from P.J.K. GmbH (Kleinblittersdorf, Germany), polyethylenimine from Polysciences (Warrington, WI). Hank's buffered salt solution (HBSS), Free-style medium and Pluronic F68 from Invitrogen (Carlsbad, CA), AMG7703 (previously known as Phenylacetamide 1) [17] was synthesized at Amgen, Inc. (South San Francisco, CA). The detailed synthetic routes, solubility properties of AMG7703, and SAR studies have been described elsewhere [18].

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2.2. Aequorin assay

CHOK1 cells (2×10^6 per dish) were seeded in 145 mm dishes and cultured with media containing DMEM-F12 and 10% FBS. The cells were incubated at 37 °C, with 5% CO_2 overnight and transfected with 5 μg pcDNA3.1 FFA2 and 5 μg pcDNA3.1 aequorin vector constructs on the following day. At 24 h after transfection, the cells were detached in PBS containing 0.25 mM EDTA, loaded with coelenterazine, and assayed as described previously [17].

2.3. Inositol phosphate accumulation assay (IP assay)

HEK293-6E cells were cultured in Free-style medium supplemented with 10% Pluronic F-68. Twenty millilitres of HEK293-6E cells (containing 1×10^6 cells/ml) was transiently transfected with 20 μg of vector DNA expressing FFA2 using 60 μl of polyethylenimine reagent (stock concentration of 1 mg/ml, pH 7.0) in a 125-ml shaker-flask. After 24 h of transfection, around 50,000 cells/well were seeded in a 96-well plate and labeled with [^{3}H]myoinositol for 16 h. The cells were then treated for 1 h at 37 °C with serial dilutions of test compounds in HBSS containing 25 mM HEPES (pH 7.4), 10 mM LiCl, and 0.01% HSA. Cells were lysed with 20 mM formic acid for 4 h at 4 °C. Ysi-SPA beads were added to the cell lysates and incubated overnight in the dark. Radioactivity was recorded on a Microbeta scintillation counter (Perkin Elmer).

2.4. Data analysis

All the results from the aequorin assay are representative of three independent experiments performed in duplicate. The data shown for IP assay are ±S.E of three independent experiments performed in duplicate. The maximal functional response is expressed as a percentage of the wild type efficacy for acetate and AMG7703. The data were fitted to a four parameter logistic equation using non-linear regression analysis using Prism 5.01 (Graph-Pad Software Inc., San Diego, CA).

2.5. Molecular modeling

Molecular modeling was carried out as previously described [17].

3. Results

3.1. Two putative binding pockets for synthetic ligand

We have previously reported the identification of a series of small molecule phenylacetamides as novel agonists for FFA2 [17,18]. A representative compound, AMG7703 (Fig. 1A), from the series was demonstrated to positively cooperate with endogenous ligands on FFA2 activation and suggested that the synthetic ligand binds at a distinct site on the receptor from orthosteric ligand [17]. To gain further insight into the binding and allosteric interactions between orthosteric and allosteric sites, we constructed a molecular model of FFA2 based on homology modeling using the X-ray crystal structure of the human β_2-adrenergic receptor as a template [17,19]. This model revealed two probable binding sites for synthetic ligand, Mode 1 (Fig. 1B and Supplementary Fig. 1A) and Mode 2 (Fig. 1C and Supplementary Fig. 1B).

3.2. Mutations in the probable Mode 1 synthetic ligand binding pockets

The putative Mode 1 binding pocket is located in a cavity between TM2, 3, 7, and EL2 of the FFA2 receptor (Fig. 1B and Supplementary Fig. 1A). The contacts between AMG7703 and the receptor, as seen in the model, are primarily hydrophobic in nature, and no strong polar contacts were observed.

Based on this model, several potentially important residues were mutated to assess their contributions to AMG7703 induced receptor activation and allosterism. The residues chosen from Mode 1 include I66, F89, L173, V259, and V260. Surprisingly, none of these mutations had any significant effects on the potency of acetate and AMG7703 in either the aequorin assay or IP accumulation assay as summarized in Table 1 and Table 2 and shown in Supplementary Fig. 2. In addition, none of the Mode 1 mutations had a selective effect on AMG7703 activity over acetate. This may suggest that either the Mode 1 pocket is not responsible for AMG7703 binding or that the pocket is flexible enough to accommodate single substitutions without significantly affecting ligand-induced receptor activation. Therefore, double and triple mutations of hydrophobic residues at positions I66, V259, and V260 were also generated to increase the potential steric hindrance between AMG7703 and the receptor. Although, I66M/V259F and I66M/V259F/V260T did result in a significant right shift in AMG7703 potency, their effects on acetate were

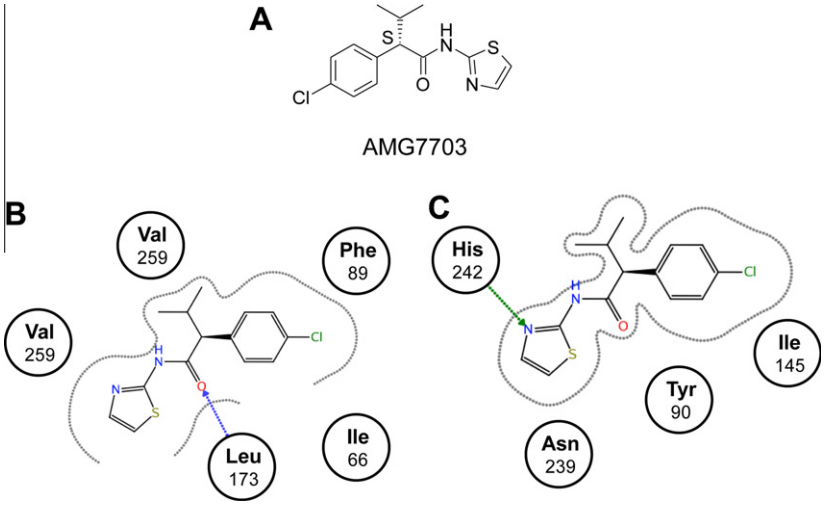

Fig. 1. Molecular models of ligand binding to FFA2. (A) Structure of allosteric agonist, AMG7703. (B) Molecular model of putative AMG7703 Mode 1 binding site on FFA2 showing residues selected for mutagenesis and corresponding electrostatic solvent accessible surfaces. (C) Putative AMG7703 Mode 2 binding site showing residues selected for mutagenesis and corresponding electrostatic solvent accessible surfaces.

Table 1
Changes in potency (log EC_{50}) of FFA2 mutants in response to acetate and AMG7703 compared with wild type receptor in the aequorin assay.

Constructs	Acetate		AMG7703	
	Log EC_{50}	Delta Log EC_{50}	Log EC_{50}	Delta Log EC_{50}
WT	2.8 ± 0.12		6.1 ± 0.06	
I66M	2.7 ± 0.1	0.1	6.0 ± 0.31	0.1
F89H	2.1 ± 0.03	0.7	5.4 ± 0.09	0.7
Y90W	Inactive		4.3 ± 0.17	1.8
H140A	1.7 ± 0.25	1.1	4.3 ± 0.12	1.8
I145F	2.8 ± 0.15		6.2 ± 0.03	−0.1
L173W	2.0 ± 0.09	0.8	5.1 ± 0.12	1.0
N239A	Inactive		4.6 ± 0.06	1.5
H242F	Inactive		4.5 ± 0.12	1.6
V259T	2.6 ± 0.12	0.2	6.0 ± 0.09	0.1
V260L	2.8 ± 0.12		5.7 ± 0.2	0.4
I66M/V259F	Inactive		4.8 ± 0.04	1.3
I66M/V259F/V260T	Inactive		4.4 ± 0.19	1.7

Table 2
Changes in potency (log EC_{50}) of FFA2 mutants in response to acetate and AMG7703 compared with wild type receptor in the IP accumulation assay.

Constructs	Acetate		AMG7703	
	Log EC_{50}	Delta Log EC_{50}	Log EC_{50}	Delta Log EC_{50}
WT	3.3 ± 0.11		5.9 ± 0.05	
I66M	3.5 ± 0.00		6.3 ± 0.06	−0.4
F89H	2.5 ± 0.1	0.8	5.3 ± 0.03	0.6
Y90W	Inactive		4.7 ± 0.06	1.2
H140A	2.4 ± 0.4	0.9	5.7 ± 0.19	0.2
I145F	3.4 ± 0.2	−0.1	6.1 ± 0.09	−0.3
L173W	2.0 ± 0.03	1.3	5.3 ± 0.11	0.6
N239A	Inactive		5.3 ± 0.12	0.6
H242F	Inactive		4.9 ± 0.05	1.0
V259T	3.2 ± 0.03	0.1	5.8 ± 0.06	0.1
V260L	3.3 ± 0.06		5.9 ± 0.03	
I66M/V259F	Inactive		5.2 ± 0.12	0.7
I66M/V259F/V260T	Inactive		4.8 ± 0.07	1.1

more pronounced, and rendered acetate inactive (Supplementary Fig. 2 and Tables 1 and 2). Since the Mode 1 pocket is near the putative acetate binding pocket described in our previous report [16], the effects of these mutations on acetate activities are likely to be an indirect effect and suggest a possible conformational change on the receptor that affected both orthosteric and allosteric sites. To our surprise, acetate activity was fully rescued on the triple-mutant receptor in the presence of varying concentrations of AMG7703 (Fig. 2A). In addition, acetate also displayed a positive cooperativity on AMG7703 activity by increasing the potency for AMG7703 in the presence of different concentrations of acetate (Fig. 2B).

To further investigate whether Mode 1 represents the actual binding site for AMG7703, we evaluated additional predictions based on this model. The molecular model suggested a potential polar contact between the amide thiazole moiety of AMG7703 and the backbone NH of L173 in the EL2 (Fig. 1B). The potential interaction between AMG7703 and R255, and between R255 and L173, as well as improved geometry for these various interactions seen in the modeled ternary complex between receptor/acetate/AMG7703, suggested L173 as a key residue for synthetic ligand/receptor interaction as well as for allosteric interaction between different binding sites. However, analysis of L173W mutation had no significant effect on AMG7703 as compared to acetate (Supplementary Fig. 2A, B, E, and F). In addition, a positive allosteric effect between acetate and AMG7703 was still observed on this mutant receptor (Fig. 2C and D). These results indicate that residues suggested by Mode 1 binding are most likely not involved in AMG7703 binding and activation of the receptor.

3.3. Mutations in the probable Mode 2 synthetic ligand binding pockets

The putative Mode 2 binding pocket is located in a cavity between TM3, 4, and 6 (Fig. 1C and Supplementary Fig. 1B). The synthetic ligand is surrounded primarily by hydrophobic residues and the thiazole amide ring of AMG7703 is proximal to H242, forming a hydrogen bond with the side chain NH (Fig. 1C). The model predicted Mode 2 as an alternative binding pocket to Mode 1 but not a simultaneous binding site.

The positions chosen from the Mode 2 binding pocket for mutagenesis studies include Y90, I145, N239, and H242. No effects of the I145F mutation were observed on either AMG7703 or acetate activity, while N239A mutation affected activities of both ligands (Tables 1 and 2, and Supplementary Fig. 3). Mutations at the Y90 and H242 positions significantly right shifted AMG7703 potency and at the same time completely abolished acetate activity (Supplementary Fig. 3B and D and Tables 1 and 2). Therefore, it is difficult to conclude from this evidence whether there is a direct binding of synthetic ligand into the Mode 2 pocket. The effects of Mode 2 mutants on allosteric interactions were also examined. Although N239A significantly reduced the response of the receptor to AMG7703 and acetate, positive cooperativity between AMG7703 and acetate was still observed (Fig. 3A and B). With respect to the H242F mutant receptor, acetate appeared to have antagonized receptor response to AMG7703 (Fig. 3C) with no effect on cooperativity to AMG7703 (Fig. 3D). These antagonistic effects were observed only at very high acetate concentrations (100–200 mM) and we could not rule out these as potential assay artifacts. However, the lack of a positive or negative cooperativity effect between acetate and AMG7703 with H242 mutant (Fig. 3C and D) suggests that H242 may be an important residue for allosteric interactions.

3.4. Mutations in H140

We have observed that mutations outside the putative acetate binding pocket affect acetate activity and some of them can be rescued by the allosteric ligand. We therefore also wanted to explore conserved residues known to affect GPCR function in acetate-induced activation of the receptor. A potentially important residue for FFA2 function is H140, which is located in TM4 and is conserved between FFA receptors. This residue has been shown to contribute to both FFA1 binding and function [14,20] but has been reported as having only a modest effect on FFA2 [15]. Similar to previous observations [15], the H140A mutation only had a modest effect in acetate potency in the aequorin and IP assays (Fig. 4 and Table 1 and 2). The effect of the H140A mutation on AMG7703 potency appeared more pronounced in the aequorin assay (~60-fold right shift in potency, Fig. 4B and Table 1) but did not significantly affect AMG7703 activity in the IP assay (Fig. 4D and Table 2). The different effects seen in the two assays may be explained by the fact that the transient aequorin assay is more sensitive to the on–off rate of the ligand. Overall, it appears that the H140A mutation has a modest effect on the functional activity of both acetate and the synthetic ligands.

4. Discussion

We have previously reported the identification of a series of phenylacetamides as selective allosteric agonists for FFA2 [17]. Based on the X-ray crystal structure of the human β_2-adrenergic

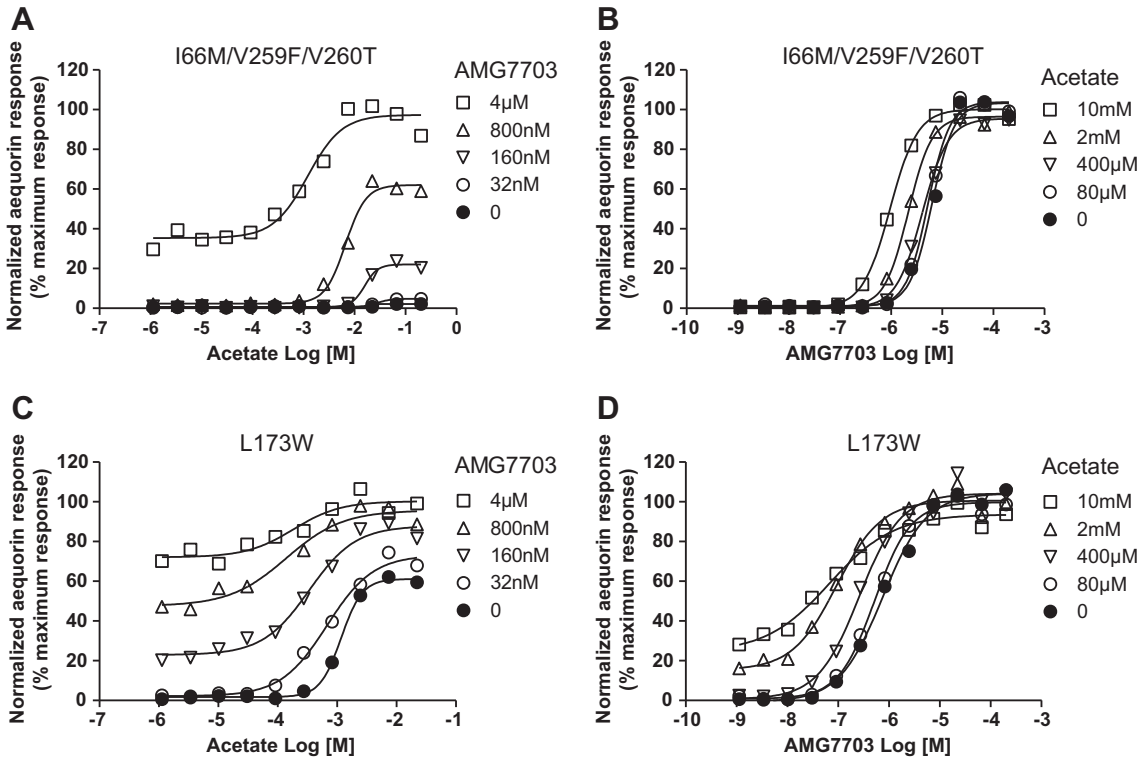

Fig. 2. Positive cooperativities are still observed on receptors with mutations in the putative Mode 1 binding pocket. Allosteric effects between acetate and AMG7703 were characterized in aequorin assay on I66M/V259F/V260T triple-mutant receptor (A and B) or L173W mutant receptor (C and D).

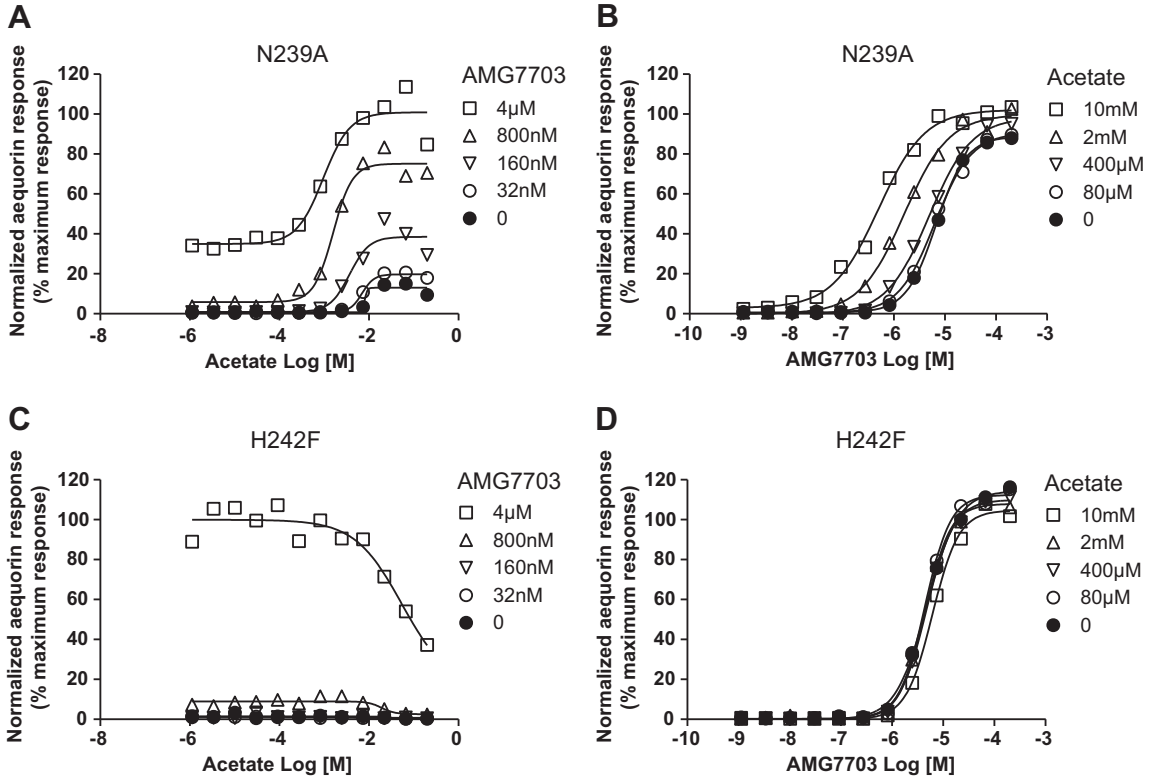

Fig. 3. Allosteric modulations between acetate and AMG7703 on receptor with mutations in the putative Mode 2 binding pocket. Allosteric effects between acetate and AMG7703 were characterized in aequorin assay on N239A mutant receptor (A and B) or H242F mutant receptor (C and D).

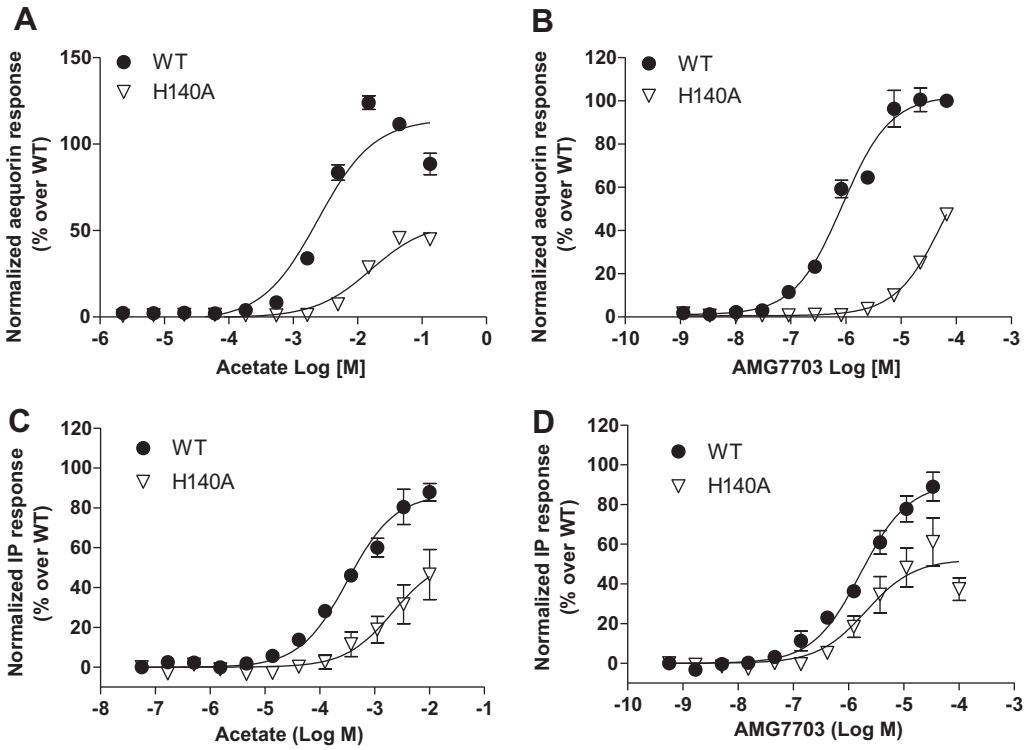

Fig. 4. Effects of H140A mutation on FFA2 activity. Wild type or mutant FFA2 receptors were transiently transfected in CHO cells for aequorin assay (A and B) or HEK293-6E cells for IP assay (C and D). (A and C) show the effects of H140A mutation on acetate activity. (B and D) show the effects of H140A mutation on AMG7703 activity.

receptor, a homology model was built for FFA2, and subsequent analysis revealed putative binding sites for phenylacetamide. In this study, we tested these putative synthetic ligand binding sites experimentally by mutagenesis to examine their contributions to ligand binding and positive cooperativity effects.

The analysis of mutations in the putative Mode 1 pocket suggests that this is not a real binding site for the synthetic ligand. Observations leading to this conclusion are, first, modeling predicted the importance of L173 of EL2 to AMG7703 binding and the positive allosteric interaction with acetate. However, mutating L173 to the bulkier tryptophan side chains did not cause any significant or selective decrease in AMG7703 potency nor did it cause any significant effects on positive cooperativity with acetate (Tables 1 and 2 and Fig. 2). Second, based on the Mode 1 model, the change of I66 to methionine (the corresponding residue in FFA3) should have caused significant steric hindrance, which was suggested as a possible explanation for the selectivity of AMG7703 for FFA2 over FFA3 [17]. However, I66M had no significant effect on AMG7703 potency (Tables 1 and 2). Although, double and triple mutations with V259 and V260 did alter AMG7703 potency, their effects on acetate binding and function were more pronounced. In addition, the model also predicted that significant structural alterations are needed on mutant receptors with bulkier single or multiple substitutions to accommodate the simultaneous binding of both acetate and AMG7703. This potential steric hindrance should have reduced both ligand binding as well as allosteric interactions. To the contrary, AMG7703 partially rescued the loss-of-function phenotype of acetate on the I66M/V259F/V260T triple mutant, and positive cooperativity was observed in both the triple and the L173W mutant receptors. Third, unlike the acetate pocket mutants [16], no mutation in the Mode 1 binding pocket generated selective effects on AMG7703 over acetate (Tables 1 and 2). Fourth, the structure activity relationship (SAR) around AMG7703 is less consistent with Mode 1 binding (unpublished results); therefore, these results suggest that Mode 1 residues are not likely to interact directly with the synthetic ligands.

The effects of the I66M/V259F/V260T triple mutant on ligand binding and allosterism is quite interesting and unexpected. This mutant completely abolished the ability of acetate to activate the receptor. Since the Mode 1 pocket is near the putative acetate binding pocket, we speculate that the triple mutation induced a conformational change on the receptor and indirectly affected the orthosteric binding pocket to prevent the endogenous ligand acetate from activating the receptor. The binding of AMG7703 was presumably able to reverse this effect through allosteric interaction, and rescued the response of the mutant receptor to acetate. The rescued response on the triple-mutant receptor to acetate was stronger than the rescued response observed with previously described mutant R255A (Fig. 2A and [16]). The difference between the two mutants may be the result of whether the mutated residues directly contribute to acetate binding. Since R255 may directly interact with the acidic head group of acetate, the loss of electrostatic interactions in the R255A mutant is unlikely to be completely compensated for by the allosteric effects from AMG7703 binding. However, the effects of the triple mutation are only conformational since they are not in the direct acetate binding pocket. Therefore, this kind of loss-of-function mutation has the potential to be fully restored with the reversal or compensatory conformational changes induced by an allosteric ligand. These results support the potential importance of allosteric agonists in a therapeutic setting and the possibility of identifying ligands that can activate seemingly inactive receptors that might occur as SNPs [21,22].

The mutational analysis of the Mode 2 pocket did not provide a clear conclusion to its contribution to synthetic ligand binding. Mutation of residue N239 located in the Mode 2 pocket to alanine did impact AMG7703 activity more than acetate in the aequorin assay (Supplementary Fig. 3, and Table 1). Another residue that

has been noted to have a selective effect on AMG7703 is H140. It has been suggested that H140 may play a role in the binding of endogenous ligands to FFA2 [15]. This histidine residue in TM4 is conserved between FFA1-3 and has been shown to contribute to binding as well as function of FFA1 [14,20]. When H140 was mutated to alanine, we found a modest shift in potency and efficacy for acetate in our functional assays (Fig. 4, Table 1 and 2) similar to the shift in potency observed previously [15]. However, the H140A mutation appeared to reduce the potency and to right shift AMG7703 concentration response curve more than acetate in the aequorin assay (Fig. 4A and B, and Table 1). Interestingly, H140 is located near the Mode 2 pocket (Supplementary Fig. 4) which may suggest the potential importance of the Mode 2 binding site for synthetic ligands. However, these effects are rather mild and other mutations in the Mode 2 pocket did not yield selective effects on AMG7703 activity. The mutation at the H242 position unexpectedly abolished acetate-induced receptor activation. This loss-of-function mutation was not rescued by AMG7703 (Fig. 3C). Although these effects were similar to the phenotype of the acetate binding pocket mutation R180A described previously [16], since H242 is not expected to form direct interactions with acetate, this suggests that H242 may play a critical role in allosteric interactions between the orthosteric and allosteric sites.

In this study, we used mutagenesis to probe the putative binding sites for synthetic ligands of FFA2 and identified residues important for positive allosteric effects as well as previous unknown residues that affect orthosteric site activity. In summary, the results indicate that the Mode 1 binding pocket is not a binding site for the synthetic ligand. In addition, residues H242 in TM6 appear critical for allosteric interaction between orthosteric and allosteric sites. Further such studies on the ability of the allosteric ligand to rescue inactive FFA2 receptors may provide a greater understanding of the dynamics of GPCR conformational states and the application of this understanding to the development of novel allosteric ligands.

Conflicts of interest statement

All authors are employed by Amgen Inc.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.12.139.

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