

THEMED ISSUE: GPCR

REVIEW

Agonism and allosterism: the pharmacology of the free fatty acid receptors FFA2 and FFA3

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The free fatty acid receptors FFA2 and FFA3 are recently de-orphanized G protein-coupled receptors that share a group of short-chain free fatty acids as endogenous ligands. The expression of FFA2 and FFA3 by immune cells, in parts of the gastro-intestinal tract and by white adipocytes has suggested their potential as therapeutic targets in conditions including inflammation and obesity. However, although FFA2 and FFA3 display distinct structure–activity relationships for stimulation by short-chain free fatty acids, the overlap between these endogenous agonists and the lack of synthetic small molecule ligands that display selectivity between these two receptors has, until recently, hindered efforts to resolve their individual functions. Recently, chloro- α -(1-methylethyl)-*N*-2-thiazolylbenzeneacetamide has been described as an FFA2 selective ago-allosteric ligand, not only being a direct agonist but also acting as a positive allosteric modulator of the function of short-chain free fatty acids at FFA2. Mutation of a pair of key arginine residues near the top of transmembrane domains V and VII of both FFA2 and FFA3 eliminates the function of short-chain free fatty acids but is without effect on the direct agonist action of chloro- α -(1-methylethyl)-*N*-2-thiazolylbenzeneacetamide at FFA2, confirming the distinct nature of the binding site of the ago-allosteric regulator from the orthosteric binding site for free fatty acids. An understanding of structure–activity relationships for ligands related to chloro- α -(1-methylethyl)-*N*-2-thiazolylbenzeneacetamide is likely to provide greater insight into the mode of action and site of binding of this ligand, but further FFA2 and FFA3 selective ligands, preferably with higher potency/affinity, will be required to fully explore the physiological function of these receptors.

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Abbreviations: eYFP, enhanced yellow fluorescent protein; FFA2/3, free fatty acid receptor 2/3; GPCR, G protein-coupled receptor; TMD, transmembrane domain

Introduction

Although G protein-coupled receptors (GPCRs) are the molecular targets for a wide range of therapeutic small molecule medicines, only a small proportion of the GPCR family have been exploited in this way (Wise *et al.*, 2004). In part,

this reflects the importance of aminergic neurotransmitters and hormones in the regulation and homeostasis of key physiological functions that may become dysregulated in disease. However, a substantial number of GPCRs with expression patterns consistent with them playing important roles in tissues and functions that are known to be subverted in disease have yet to be exploited effectively. In a number of cases, this reflects a lack of knowledge of the endogenous modulators that regulate these receptors and such receptors are classified, therefore, as 'orphans' (Wigglesworth *et al.*, 2006). A series of biomolecules, generally considered as metabolic intermediates, have recently been shown to act as agonists at certain, previously 'orphan', GPCRs. These include β -hydroxybutyrate (Taggart *et al.*, 2005) [and/or butyrate (Thangaraju *et al.*, 2009)] as an activator of GPR109A, aromatic D-amino acids as chemoattractant factors activating

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GPR109B (Irukayama-Tomobe *et al.*, 2009), lactate as an agonist of GPR81 (Cai *et al.*, 2008; Liu *et al.*, 2009), kynurenic acid as an agonist of GPR35 (Wang *et al.*, 2006a), succinate as an agonist of SUCNR1 (previously designated GPR91) (He *et al.*, 2004) and oxaloacetate as an agonist of the OXGR1 receptor (previously designated GPR99) (He *et al.*, 2004). Furthermore, free fatty acids of various chain length and degree of saturation have been shown to act as agonists at both GPR120 (Hirasawa *et al.*, 2005) and GPR84 (Wang *et al.*, 2006b) as well as the group of related GPCRs named free fatty acid receptors FFA1, FFA2 and FFA3 (previously designated GPR40, GPR43 and GPR41 respectively) (Briscoe *et al.*, 2003; Brown *et al.*, 2003; Itoh *et al.*, 2003; Kotarsky *et al.*, 2003; Le Poul *et al.*, 2003; see Stoddart *et al.*, 2008a for review). In a number of these cases, identification of endogenous ligands able to activate these GPCRs has not yet resulted in the identification (or at least the public disclosure) of synthetic, small molecule ligands able to act as agonists or antagonists of these receptors.

Phylogenetic analysis has clustered FFA1-3 into a branch of the rhodopsin-like, class A GPCRs that contains receptors for phospholipids, nucleotides, citric acid cycle intermediates and the protease-activated receptors, along with a number of orphan GPCRs (Surgand *et al.*, 2006; Tikhonova *et al.*, 2007). FFA1 has attracted considerable attention as a potential therapeutic target for the treatment of diabetes, based predominantly on its expression in pancreatic β -cells and the known role of medium- and longer-chain fatty acids, that are agonists at this GPCR, in modulating glucose-dependent release of insulin (Milligan *et al.*, 2006; Stoddart *et al.*, 2008a). This has resulted in the identification of a number of small molecule agonist and antagonist ligands at this receptor (Briscoe *et al.*, 2006; Garrido *et al.*, 2006; Bharate *et al.*, 2008; Christiansen *et al.*, 2008; Tan *et al.*, 2008; Tikhonova *et al.*, 2008; Humphries *et al.*, 2009). A considerable number of recent reviews have centred on the function and pharmacology of this receptor and whether short-term stimulation or longer-term blockade might result in the most effective therapeutic treatment (Milligan *et al.*, 2006; Brownlie *et al.*, 2008; Costanzi *et al.*, 2008; Hirasawa *et al.*, 2008; Swaminath, 2008; Telvekar and Kundaikar, 2008). Significantly less discussion of the pharmacology and function of the related family members FFA2 and FFA3 is available and these receptors will, therefore, be the focus of the current review.

De-orphanization and modes of signal transduction of FFA2 and FFA3

Fatty acids with chain length of less than six carbons are endogenous agonists for FFA2 and FFA3 (Brown *et al.*, 2003; Le Poul *et al.*, 2003; Nilsson *et al.*, 2003) but there are distinct chain length structure-activity relationships at the two receptors with FFA2 displaying potency $C2 = C3 > C4 > C5 = C1$ and FFA3 $C3 = C4 = C5 > C2 = C1$ (Figure 1). Brown *et al.* (2003) used recombinant cell systems to demonstrate that FFA2 was activated by the carboxylic acid, acetate and related molecules. This was also noted by Le Poul *et al.* (2003) and Nilsson *et al.* (2003). Brown *et al.* (2003) and Le Poul *et al.* (2003) also demonstrated that short-chain fatty acids were agonists at FFA3.

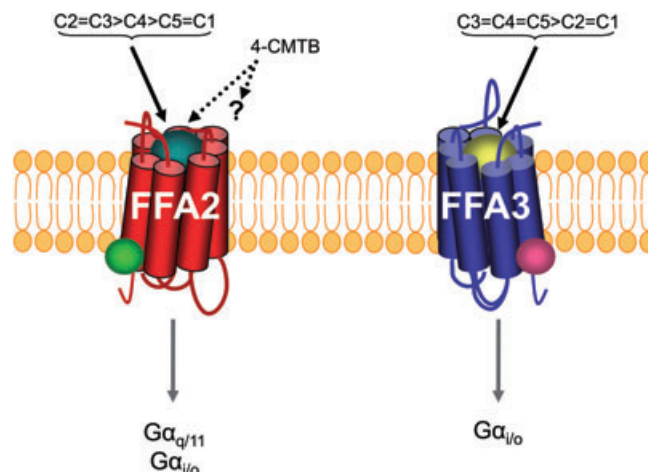


Figure 1 Ligand selectivity and G protein-coupling profiles of FFA2 and FFA3. Cartoons of the receptors FFA2 (red) and FFA3 (blue) are shown. The relationship of chain length (C) of free fatty acids as orthosteric agonists (implied by the teal or gold coloured balls) is shown for each receptor as is the potential for allosteric regulators of each receptor (green and pink coloured circles). chloro- α -(1-methylethyl)-N-2-thiazolylbenzeneacetamide (4-CMTB) is the first described selective modulator of FFA2 and it is both a direct agonist and a positive allosteric modulator of the action of short-chain free fatty acids (Lee *et al.*, 2008). The molecular basis of interaction of 4-CMTB with FFA2 and its selectivity for FFA2 over FFA3 remains to be defined. FFA2/3, free fatty acid receptor 2/3.

Despite responding to the same group of endogenous agonists, FFA2 and FFA3 have distinct G protein-coupling specificities (Figure 1). FFA2 couples to both pertussis toxin-sensitive and -insensitive G proteins (Brown *et al.*, 2003; Le Poul *et al.*, 2003; Nilsson *et al.*, 2003; Stoddart *et al.*, 2008b) while FFA3 couples selectively to pertussis toxin-sensitive $G\alpha_i$ family G proteins (Brown *et al.*, 2003; Le Poul *et al.*, 2003; Stoddart *et al.*, 2008b). However, as with many $G\alpha$ -coupled GPCRs (Kostenis *et al.*, 2005), Le Poul *et al.* (2003) found that co-expression with the promiscuous G protein, $G\alpha_{i6}$ (Milligan and Kostenis, 2006), allowed activation of FFA3 to be coupled to the release of $[Ca^{2+}]_i$, despite an inability to couple directly to $G\alpha_{q/11}$. Based on these observations, Stoddart *et al.* (2008b) employed a chimeric Gly⁶⁶Asp $G\alpha_{q-15}$ variant (Heydorn *et al.*, 2004) to link activation of FFA3 to the elevation of intracellular $[Ca^{2+}]$ and explore the structure-activity relationships of short-chain free fatty acids at this receptor. Furthermore, given the pleiotropic effects of short-chain free fatty acids, Stoddart *et al.* (2008b) also employed cells able to express either FFA2 or FFA3 in an entirely inducible manner (Figure 2) to ensure that effects of the ligands were actually mediated via the receptor.

Identification, tissue distribution and physiological function of FFA2 and FFA3

The genes encoding FFA2 and FFA3 in man were first identified by Sawzdargo *et al.* (1997) within a tandemly encoded group of intronless genes, located at chromosome 19q13.1. This region also encompasses the genes encoding FFA1 and GPR42. The proteins predicted from these sequences contained seven

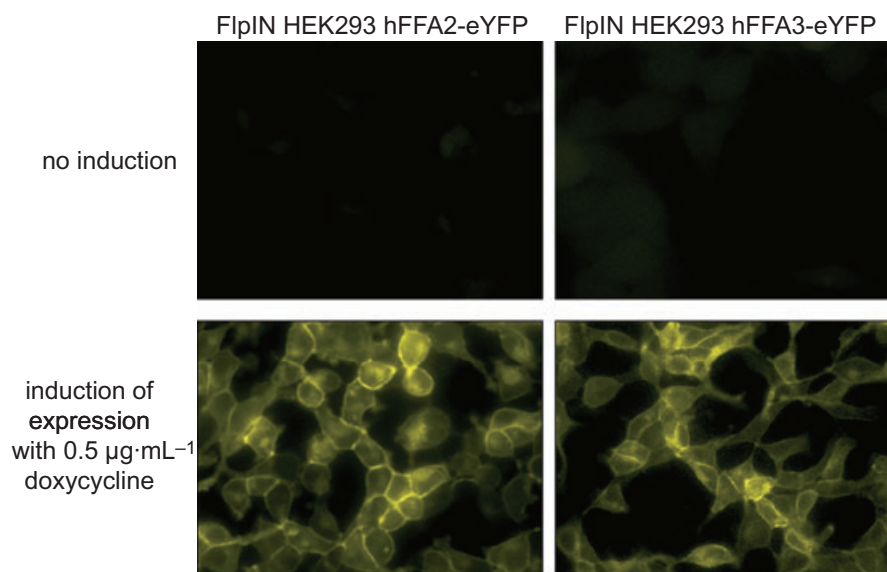


Figure 2 Cell lines able to express FFA2 or FFA3 in an inducible manner. Short-chain fatty acids such as acetate and propionate are pleiotropic in action and many effects in cells are likely to be mediated via non-receptor mechanisms. To define FFA2- and FFA3-specific effects of such ligands, Stoddart *et al.* (2008b) established Flp-In TREx HEK293 cell lines able to express C-terminally eYFP tagged forms of human FFA2 (left hand panels) or FFA3 (right hand panels) in an antibiotic dependent fashion. In the absence of doxycycline (top panels) the receptors cannot be detected but after treatment with doxycycline (bottom panels) the characteristic pattern of cell surface location is observed. Actions of short-chain fatty acids produced without treatment of the cells with doxycycline were considered unspecific. eYFP, enhanced yellow fluorescent protein; FFA2/3, free fatty acid receptor 2/3.

putative transmembrane domains (TMDs) and a variety of other features common to class A GPCRs. Although having 98% homology with FFA3 (Sawzdargo *et al.*, 1997), GPR42 has been suggested to be a pseudogene in man (Brown *et al.*, 2003). However, very recent studies involving complete gene sequencing and analysis of human genome public databases have suggested that the six amino acid differences between FFA3 and GPR42 are actually polymorphisms rather than gene-specific differences and that a substantial number of individuals are likely to express functional GPR42 (Liaw and Connolly, 2009). FFA2 mRNA can be detected in a variety of tissues but highest expression is found in immune cells such as neutrophils, monocytes, peripheral blood mononuclear cells, B-lymphocytes and polymorphonuclear cells (Brown *et al.*, 2003; Le Poul *et al.*, 2003; Nilsson *et al.*, 2003). Considerable levels of FFA2 mRNA were also detected in bone marrow and spleen but this probably reflects expression of the receptor by immune cells (Le Poul *et al.*, 2003). FFA2 mRNA has also been detected in eosinophils (Nakajima *et al.*, 2004). Nilsson *et al.* (2003) found expression of the receptor in skeletal muscle and heart, while FFA2 mRNA has also been reported in adipose tissue (Hong *et al.*, 2005; Ge *et al.*, 2008) and rat distal ileum and colon (Karaki *et al.*, 2006). FFA3 has a more widespread expression pattern than FFA2. Initial studies on FFA3 detected high levels of receptor mRNA in a range of human tissues with highest levels of expression in adipose tissue (Brown *et al.*, 2003). Substantial expression was also detected in pancreas, spleen, lymph nodes, bone marrow and peripheral blood mononuclear cells (Brown *et al.*, 2003; Le Poul *et al.*, 2003). The expression of FFA3 in adipose cells has been confirmed (Xiong *et al.*, 2004). Potential roles for FFA2 and FFA3 have recently been reviewed extensively (Stoddart *et al.*, 2008a) and will not be covered here in detail. FFA2 may

mediate the known effects of short-chain free fatty acids on leukocyte chemotaxis and movement towards sites of bacterial infection. Furthermore, using an FFA2 knockout mouse model, Ge *et al.* (2008) found that neither acetate nor propionate was able to inhibit lipolysis in primary adipocytes isolated from the FFA2^{-/-} mouse, suggesting that FFA2 is able to inhibit lipolysis and, therefore, regulate plasma free fatty acid levels. However, based on the known effects of sodium propionate to increase circulating leptin levels in mice (Xiong *et al.*, 2004) and that FFA3 knock-down has been reported to almost completely abolish the ability of propionate to induce leptin reporter expression and consequently leptin production (Xiong *et al.*, 2004), FFA3 may also play an important role in signalling adipocyte mass and lipid stores. Short-chain free fatty acids such as acetate, propionate and butyrate are both a primary source of energy and also regulate development, insulin and glucagon secretion, and other physiological processes in ruminants such as cattle and sheep. It is also of note, therefore, that bovine forms of FFA2 and FFA3 have recently been cloned and shown to respond to these ligands (Wang *et al.*, 2009). As both receptors have similar pharmacological profiles, the availability of selective agonists and antagonists would be invaluable in dissecting the relative contribution of each receptor in the normal physiology of adipocytes and in providing further support for conclusions reached from knockout and knock-down studies.

Structural features of FFA2 and FFA3 and the binding of free fatty acids

Both FFA2 and FFA3 are more closely related to each other than to any other GPCR. Despite this, they exhibit only 43%

overall similarity (Stoddart *et al.*, 2008a). As with the vast majority of class A GPCRs, both FFA2 and FFA3 contain cysteine residues at the top of the third TMD and in the second extracellular loops that are predicted to contribute to structure via the formation of intra-molecular disulphide bonds, although this remains to be demonstrated experimentally. They also contain an arginine at the bottom of TMD III within the highly conserved Asp-(Glu)-Arg-Tyr (D(E)RY) domain of class A GPCRs. FFA2 contains a Glu-Arg-Tyr motif in this location, whereas in FFA3 it is a Glu-Arg-Phe motif.

The orthosteric binding pocket

Both FFA2 and FFA3 respond to short-chain free fatty acids with carbon chain length C1 (formate) to C5 (valerate) (Brown *et al.*, 2003; Stoddart *et al.*, 2008b). However, the potency of these ligands is low, within the high μM to low mM range. Because of the lack of high affinity ligands that interact with FFA2 and/or FFA3, identification of residues that contribute to the binding of orthosteric ligands in these two GPCRs has so far been inferred simply from loss of function or impairment of signalling of point mutants of these receptors (Stoddart *et al.*, 2008b) or from modelling studies (Lee *et al.*, 2008). In initial studies designed to explore the contributions of the six amino acids predicted to differ between human FFA3 and the non-functional GPR42 sequence, Brown *et al.* (2003) systematically inter-converted each variant residue. Alteration of Arg¹⁷⁴ of FFA3, that is predicted to be located in extracellular loop 2, to the corresponding Trp residue of GPR42 generated an FFA3 mutant that was no longer able to respond to the C3 fatty acid propionate. Conversely, introduction of Arg¹⁷⁴ into GPR42 rendered the previously unresponsive protein able to generate signals to propionate (Brown *et al.*, 2003). Alteration of the residue equivalent to Leu²²⁷ (residue 6.37 in the nomenclature of Ballesteros and Weinstein, 1995) of human FFA2 to Val at the bottom of TMD VI in the rat orthologue of FFA2 resulted in constitutive signalling (Brown *et al.*, 2003). This region is often implicated in GPCR activation of G proteins.

Based on both the lack of function of fatty acid amides at each of FFA1-3 (Briscoe *et al.*, 2003; Brown *et al.*, 2003) and the loss of function of longer-chain fatty acids at forms of FFA1 in which the Arg residues at 5.39 and 7.35 were mutated (Sum *et al.*, 2007), Stoddart *et al.* (2008b) aligned human FFA1, FFA2 and FFA3 and noted the conservation of these two residues. Mutation of Arg 5.39 in FFA2 to a range of amino acids, including Lys, resulted in loss of capacity of the C2 and C3 fatty acids acetate and propionate to cause elevation of intracellular Ca^{2+} in HEK293 cells transfected to express these variants transiently (Stoddart *et al.*, 2008b). Because it was unclear in such studies if the mutated forms of FFA2 were able to reach the cell surface efficiently, Stoddart *et al.* (2008b) established stable HEK293 cell lines able to express C-terminally enhanced yellow fluorescent protein (eYFP)-tagged forms of either wild-type FFA2 or Arg 5.39 Ala FFA2, only upon addition of the antibiotic doxycycline (Figure 2). Imaging of such induced cells demonstrated similar sub-cellular distribution of the two forms and confirmed that Arg 5.39 Ala FFA2 was unable to respond to either acetate or

propionate (Stoddart *et al.*, 2008b). These cells had the additional benefit of providing proof that induction of expression of FFA2 was required for the short-chain free fatty acids to elevate intracellular Ca^{2+} and that this was not a non-specific effect of the ligands (Stoddart *et al.*, 2008b). Construction of HEK293 cells able to express Arg 7.35 Ala FFA2-eYFP in an inducible fashion also demonstrated the importance of this residue for the function of acetate and propionate (Stoddart *et al.*, 2008b). As noted earlier, FFA3 couples predominantly to pertussis toxin-sensitive G proteins of the G_i/G_o subfamily (Brown *et al.*, 2003; Le Poul *et al.*, 2003). Generation and inducible expression of Arg 5.39 Ala FFA3-eYFP and Arg 7.35 Ala FFA3-eYFP also demonstrated the importance of these residues in FFA3 as propionate was unable to increase binding of [³⁵S]GTP γ S in membranes from such cells whereas a robust, concentration-dependent increase was produced following induction of wild-type FFA3-eYFP (Stoddart *et al.*, 2008b). Molecular modelling, based on the structure of bovine rhodopsin, indicates the likely proximity of Arg 5.39 and Arg 7.35, as well as His 6.55, in both FFA2 and FFA3 and their importance in binding and function of the short-chain free fatty acids (Figure 3).

As with many GPCRs (Crespo and Gutkind, 2004), both FFA2 and FFA3 are able to cause phosphorylation and activation of the ERK1/2 MAP kinases (Stoddart *et al.*, 2008b). Phosphorylation of these kinases in response to both acetate and propionate in HEK293 cells induced to express variants of FFA3 was again lacking for Arg 5.39 Ala FFA3-eYFP and Arg 7.35 Ala FFA3-eYFP and, in agreement with the concept that FFA3 couples selectively to G_i/G_o G proteins, this effect was abolished by pretreatment of the cells with Pertussis toxin (Stoddart *et al.*, 2008b), which blocks GPCR-mediated activation of such G proteins by catalysing ADP-ribosylation of the cysteine residue located four amino acids from the C-terminus of the α subunit of members of this group (Milligan, 1988). By contrast, FFA2-mediated phosphorylation of the ERK1/2 MAP kinases was shown to be transduced by a combination of G_i/G_o and G_q/G_{11} G proteins, as pretreatment with a mixture of Pertussis toxin and the selective G_q/G_{11} inhibitor YM-254890 (Takasaki *et al.*, 2004) was required to ablate agonist function. However, as in Ca^{2+} elevation assays, both Arg 5.39 Ala FFA2 and Arg 7.35 Ala FFA2 failed to induce phosphorylation of the ERK1/2 MAP kinases in response to either acetate or propionate (Stoddart *et al.*, 2008b). It seems reasonable, therefore, to indicate that the carboxylate of short-chain fatty acids interacts with these receptors in a similar manner to that of the longer-chain free fatty acids with FFA1 (Sum *et al.*, 2007; Smith *et al.*, 2009a).

His (4.56) is conserved between FFA1-3 and has been shown to contribute to binding/function in FFA1 (Sum *et al.*, 2007; Smith *et al.*, 2009a). Furthermore, although not conserved in FFA2/3, the polarity of Asn 6.55 in FFA1 is retained in FFA2 and FFA3 as both have a His at this position. Alteration of His 6.55 to Ala in FFA2 essentially eliminated the ability of short-chain free fatty acids to elevate [Ca^{2+}]_i and greatly reduced the ability of the ligands to promote phosphorylation of the ERK1/2 MAP kinases (Stoddart *et al.*, 2008b). For FFA3, a His 6.55 Ala mutant was unable to respond to short-chain free fatty acids in any end point assessed, including stimulation of [³⁵S]GTP γ S binding, ERK1/2 MAP kinase phosphorylation or

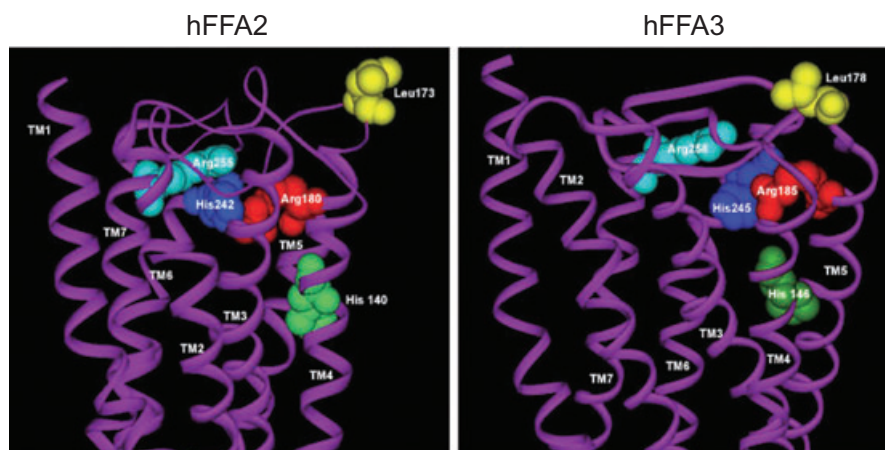


Figure 3 The orthosteric binding pocket of FFA2 and FFA3. Molecular models of human FFA2 (left) and FFA3 (right) were constructed as in Stoddart *et al.* (2008b) with the predicted carbon chain backbone of the TMDs shown in purple. Amino acids conserved between FFA2 and FFA3 (Arg 5.39, red, His 6.55, blue, Arg 7.35, cyan) that are important contributors to the binding and function of short-chain free fatty acids are shown filling the anticipated space. His 4.56 (green) plays a modulatory role that depends on the fatty acid chain length and the activity measured. Each amino acid is numbered relative to the start codon. Leu¹⁷³ (yellow) in FFA2 is located in the predicted the second extracellular loop and has been suggested to be important in binding ago-allosteric phenylacetamides (Lee *et al.*, 2008) that are selective for FFA2 over FFA3. The corresponding Leu¹⁷⁸ (yellow) in FFA3 is also shown. FFA2/3, free fatty acid receptor 2/3; TMD, transmembrane domain.

when Ca²⁺ signals were evoked by the co-expression of a chimeric G_q-G_i G protein (Stoddart *et al.*, 2008b). Effects of mutation of His 4.56 to Ala were less dramatic. For FFA2, this resulted in an approximately 10-fold reduction in the potency of both acetate and propionate in measures of [Ca²⁺]_i. Interestingly, for FFA3 the effect of the His 4.56 Ala mutant was end point-dependent. In measures of G_i-activation recorded in [³⁵S]GTPγS binding studies, propionate was entirely unable to generate a response. However, although an artificial assay, when co-expressed with a chimeric G_q-G_i G protein, propionate was able to cause elevation of [Ca²⁺]_i in single cell imaging studies via His 4.56 Ala FFA3, although only a single concentration of the agonist was tested and thus no potency information was provided (Stoddart *et al.*, 2008b). These studies indicate that a similar orientation of the carboxylate group of the endogenous ligands of FFA2 and FFA3 as suggested for FFA1 (Sum *et al.*, 2007) is likely with salt bridge interactions between the carboxylate anion of the ligand and guanidinium cation groups of Arg 5.39 and Arg 7.35 providing co-ordination (Figure 3). Indeed, such an interaction has been modelled by Lee *et al.* (2008). This is in agreement with other studies in which methyl substitutes of long-chain free fatty acids have reduced or lack activity at FFA1 (Itoh *et al.*, 2003; Salehi *et al.*, 2005) and the lack of activity of acetamide and propionamide at FFA2 and FFA3 (Stoddart *et al.*, 2008b). How chain length selectivity between FFA2 and FFA3 and, in a more general sense, between FFA2/3 and FFA1 is defined remains to be explored. In modelling studies, Lee *et al.* (2008) suggest that the lipophilic chain of propionate is in a gauche conformation and oriented towards the Ser residue at position 6.54. However, no experimental studies have been reported in which this amino acid has been modified and, as it is conserved between FFA2 and FFA3, this presumably cannot underlie the difference in structure–activity relationships for short-chain free fatty acids at the two receptors. Interestingly, in His 4.56 Ala FFA2, a gain of function to the 6 carbon free fatty acid caproate was observed

in both [Ca²⁺]_i signalling and ERK1/2 MAP kinase assays whereas this was not observed for His 4.56 Ala FFA3 (Stoddart *et al.*, 2008b). Given that free fatty acids with chain length >3 are less potent at FFA2 while potency at FFA3 is essentially the same for chain length 3–5, this observation may also provide insight into differences in the orthosteric binding pocket of FFA2 and FFA3 (Figure 3). However, further work remains to be performed. Furthermore, although both GPR84 (Wang *et al.*, 2006b) and GPR120 (Hirasawa *et al.*, 2005) are reported as receptors responsive to free fatty acids, they have no obvious homology with the FFA1-3 group and, therefore, presumably must bind ligands in a different manner. No direct studies have explored this issue but following identification of GPR120 selective agonists containing a carboxylate moiety, Suzuki *et al.* (2008) have suggested Arg 99 at the top of TMDII as a potential residue that might co-ordinate the acid. There are currently few small molecule ligands reported with selectivity between FFA1 and GPR120 (Briscoe *et al.*, 2006; Suzuki *et al.*, 2008) and thiazolidinedione peroxisome proliferator-activated receptor γ agonists such as ciglitazone and troglitazone activate both receptors, despite the limited overall sequence similarity of these two receptors.

An allosteric binding site in FFA2

A major limitation in efforts to study the biology and function of FFA2 and FFA3 is that, to date, there are no reported synthetic, orthosteric small molecule ligands. Recently, however, Lee *et al.* (2008) reported a pair of phenylacetamides as selective ‘allosteric agonists’ for FFA2. In a range of assays, including inhibition of cAMP levels and elevation of [³⁵S]GTPγS binding, these ligands acted as direct agonists at FFA2, with between 100- and 1000-fold higher potency than acetate or propionate. They were also able to inhibit lipolysis in differentiated 3T3L1 cells (Lee *et al.*, 2008), a standard, well-characterized cell-based adipocyte model. Furthermore,

the phenylacetamides also acted as positive allosteric modulators of the function of short-chain free fatty acids, able to enhance the functional potency of acetate and propionate, although the estimated allosteric co-operativity factor was modest (Lee *et al.*, 2008). However, such observations are consistent with the phenylacetamides binding to a distinct allosteric site (Langmead and Christopoulos, 2006) or potentially acting as bitopic or dualsteric ligands, molecules that have the potential to bind to multiple distinct sites on a single protein (Valant *et al.*, 2008; Antony *et al.*, 2009). Although no mutagenesis studies were performed, modelling studies were employed to suggest the location of this allosteric site. The models predict a contribution from Leu¹⁷³, located in the second extracellular loop (Figure 3), and in differing models, contributions from Arg 7.35 of the orthosteric binding site that, as noted earlier, is central to the binding/function of the short-chain fatty acids. A series of hydrophobic residues located in TMDs II, III, VI and VII were also suggested to contribute to the binding of the *p*-chlorophenyl element of the ligands. Recently, Smith *et al.* (unpublished observations) have begun to explore this issue experimentally, taking advantage of the orthosteric binding pocket mutants studied by Stoddart *et al.* (2008b). Although able to confirm both the direct agonist and positive allosteric effects of chloro- α -(1-methylethyl)-*N*-2-thiazolylbenzeneacetamide, as well as demonstrating the selectivity of this ligand for FFA2 over FFA3 and FFA1, Smith *et al.* (unpublished observations) were unable to provide evidence to support a key role for Arg 7.35 in the recognition/function of this ligand because chloro- α -(1-methylethyl)-*N*-2-thiazolylbenzeneacetamide was at least as potent and efficacious in causing inhibition of cAMP production at Arg 7.35 Ala FFA2 as at the wild-type receptor. The second extracellular loop differs in length and is relatively diverse between FFA2 and FFA3 and such loops present a substantial challenge to model. However, at least based on a simple alignment, Leu¹⁷³ in FFA2 is conserved as Leu¹⁷⁸ in FFA3 (Figure 3), although in each receptor charged amino acids located close to this residue are not well conserved. As Sum *et al.* (2009) have recently provided evidence that negatively charged amino acids in extracellular loop 2 play an important role in generating an inactive state of FFA1 by co-ordinating Arg 5.39 and Arg 7.35, it may be that similar interactions contribute to the ground state of FFA2 and FFA3, but this remains to be tested. Selective ligands for FFA3, whether orthosteric or ago-allosteric, would be of great value to understanding the biology and function of this receptor.

Conclusions

Although there is a distinct structure–activity relationship for activation of FFA2 and FFA3 by fatty acids of carbon chain length 2–5, there is insufficient selectivity to use these ligands to define clearly the function these two receptors, particularly in tissues in which they are co-expressed. Until recently, insights have been restricted to studies employing siRNA-mediated knockdown of expression and/or the generation of knockout lines of mice. Despite these approaches, the specific functions of FFA2 and FFA3 remain uncertain and pharmacological tools able to activate or block each receptor selectively

are required. The recent identification of a pair of phenylacetamides as selective ligands at FFA2 that act as both second-site agonists and positive allosteric modulators of the endogenous free fatty acids at this receptor is a helpful first step, but much remains to be achieved.

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Conflict of interest

The authors state no conflict of interest.

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