International Union of Pharmacology. LXXI. Free Fatty Acid Receptors FFA1, -2, and -3: Pharmacology and Pathophysiological Functions

LEIGH A. STODDART, NICOLA J. SMITH, AND GRAEME MILLIGAN

*Molecular Pharmacology Group, Neuroscience and Molecular Pharmacology, Faculty of Biomedical and Life Sciences,
University of Glasgow, Glasgow, Scotland, United Kingdom*

Abstract——Identification of G protein-coupled receptors that are activated by free fatty acids has led to considerable interest in their pharmacology and function because of the wide range of normal physiology and disease states in which fatty acids have been implicated. Free fatty acid receptor (FFA) 1 is activated by medium- to long-chain fatty acids and is expressed in the insulin-producing β-cells of the pancreas. Activation of FFA1 has been proposed to mediate fatty acid augmentation of glucose-stimulated insulin secretion although it is unclear whether the known long-term detrimental effects of β-cell exposure to high levels of fatty acids are also mediated through this receptor. The related receptors FFA2 and FFA3 are both activated by short-chain fatty acids although they have key differences in the signaling pathways they activate and tissue expression pattern. The aim of this review is to provide a comprehensive overview of the current understanding of the pharmacology and physiological role of these fatty acid receptors.

I. Introduction

Fatty acids have long been recognized for the variety of their effects in the body yet, until recently, these actions were thought to be mediated exclusively via actions on cellular metabolism. Thus, the recent identification and deorphanization of the free fatty acid (FFA[1]) (http://www.iuphar-db.org/GPCR/ChapterMenu

[1] Abbreviations: FFA, free fatty acid; GPCR, G protein-coupled receptor; TM, transmembrane; SCFAs, short-chain fatty acids; $[Ca^{2+}]_i$, intracellular calcium concentration; LCFAs, long-chain fatty acids; MCC-555, 5-[[6-[(2-fluorophenyl)methoxy]-2-naphthalenyl]-methyl]-2,4-thiazolidinedione; MEDICA 16, β,β'-tetramethylhexadecanedioic acid; GSIS, glucose-stimulated insulin secretion; $[^{35}S]GTP\gamma S$, guanosine 5′-O-(3-$[^{35}S]$thio)triphosphate; GW1100, (ethyl 4-[5-{[2-(ethyloxy)-5-pyrimidinyl]methyl}-2-{[(4-fluorophenyl) methyl]thio}-4-oxo-1(4H)-pyrimidinyl] benzoate); PPARγ, peroxi-

Forward?chapterID=1331) family of G protein-coupled receptors (GPCRs) has prompted reevaluation of the mechanism of action of FFAs in health and disease. Given that GPCRs are among the most tractable group of drug targets and drugs that target this family of proteins constitute more than 30% of current pharmaceuticals (Wise et al., 2004), the possibility of developing high-affinity synthetic ligands targeting members of the FFA receptor family to treat a number of multifaceted conditions, such as type 2 diabetes and inflammation, has prompted considerable interest in this receptor family. Here we describe the discovery, deorphanization, and currently recognized pharmacology of the receptor family and discuss the potential physiological and pathophysiological ramifications of FFA receptor signaling.

II. Cloning of cDNA for Free Fatty Acid Receptors 1, 2, and 3

The genes encoding FFA1, FFA2, and FFA3, originally termed *GPR40*, *GPR43,* and *GPR41*, respectively, were identified by Sawzdargo et al. (1997) as a group of intronless genes coded in tandem and located, in humans, at chromosome 19q13.1. The receptors have been given a variety of names in the literature that are summarized, along with the key features of the receptors, in Table 1. A further gene that shares 98% homology with FFA3 was also identified and termed *GPR42* (Sawzdargo et al., 1997). The genes were identified during a search for novel galanin receptors using sets of degenerate primers based on conserved sequences in human and rat galanin receptors. The proteins predicted from the gene sequences contained seven putative transmembrane domains, which, along with other features common to class A GPCRs and without the knowledge of their activating ligands, led them to be classified as orphan GPCRs. Mouse FFA2 was cloned from leukemia inhibitory factor-stimulated M1 myeloid leukemia cells (Senga et al., 2003), and cDNA homologous to human FFA3 was cloned from rat lung (Bonini et al., 1997).

III. Free Fatty Acid Receptor Structure

Hydropathy analysis of the three receptors indicates that they contain seven hydrophobic regions, consistent with transmembrane (TM) spanning helices, and further sequence analysis of FFA1, FFA2, and FFA3 allows them to be classified as belonging to the class A group of GPCRs. As with the vast majority of class A GPCRs, the three FFA receptors contain cysteine residues in the first and second extracellular loops that are likely to contribute to structure via the formation of intramolec-

some proliferator-activated receptor γ; GW9508X, 3-[4-({3-(phenyloxy)phenyl]methyl}amino)phenyl]propanoic acid; siRNA, small interfering RNA; 2BrP, 2-bromo palmitate; CPT-1, carnitine palmitoyltransferase; STZ, streptozotocin; GLP-1, glucagon-like peptide-1; GIP, glucose-dependent insulinotropic polypeptide; IBD, irritable bowel disease.

ular disulfide bonds, proline residues in TM domains 5, 6, and 7, an asparagine in TM1, and an aspartate in TM2. They also contain an arginine at the bottom of TM3, which is within the highly conserved Asp-(Glu)-Arg-Tyr motif. FFA3 contains a Glu-Arg-Tyr motif in this location, whereas in FFA1 a Gly-Arg-Tyr triplet is found and in FFA2 it is a Glu-Arg-Phe motif. In FFA1 and FFA2, two *N*-glycosylation consensus sequences (Asn-x-Ser/Thr) can be identified in extracellular loop 2 but only one is found in FFA3. The key structural features of FFA1 are exemplified in Fig. 1.

FFA1, FFA2, and FFA3 represent a family of receptors because they are more closely related to each other than to any other known GPCR. Despite this, the family exhibits somewhat limited similarity: 43% between FFA2 and FFA3 and 33 and 34% when FFA1 is compared with FFA2 and FFA3, respectively (Table 1). FFA3 and the predicted GPR42 protein share some 98% homology, differing in only six amino acids (corresponding to seven nucleotides). Only one gene corresponding to FFA3/GPR42 can be identified in the mouse and bovine genomes (Brown et al., 2003), and GPR42 is now generally thought to be an open reading frame pseudogene (Brown et al., 2005). On the basis of sequence analysis, the next most closely related receptors are the protease-activated receptors, although the FFA receptors lack the long N-terminal extracellular domain, which is cleaved in protease-activated receptors to cause receptor activation (Brown et al., 2003). Recent phylogenetic analysis has clustered FFA1–3 into a branch of class A 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).

IV. Deorphanization of Free Fatty Acid Receptors and Modes of Signal Transduction

A. Free Fatty Acid Receptor 1

All three FFA receptors remained classified as orphans until 2003 when three articles that identified a range of medium- and long-chain saturated and unsaturated fatty acids as ligands for FFA1 were published independently (Briscoe et al., 2003; Itoh et al., 2003; Kotarsky et al., 2003), and an additional three articles showed that FFA2 and FFA3 were both activated by short-chain fatty acids (SCFAs) (Brown et al., 2003; Le Poul et al., 2003; Nilsson et al., 2003). Briscoe et al. (2003) identified an unsaturated fatty acid, elaidic acid (C18:2), as the only compound from a large library to act as an agonist at FFA1 in a fluorescence imaging plate reader-based assay system that monitors the release of intracellular calcium ($[Ca^{2+}]_i$). They went on to demonstrate that more than 40 different medium and long-chain fatty acids (LCFAs) were able to activate FFA1 with micromolar range potency and, of these, eicosatrienoic acid (C20:3) was the most potent with a pEC_{50} of

TABLE 1
Current human free fatty acid receptor nomenclature and key features

Receptor Name	Human Free Fatty Acid Receptors		
	FFA1	FFA2	FFA3
Previous names	GPR40, FFAR1	GPR43, FFAR2	GPR41, FFAR3
Gene location	19q13.1	19q13.1	19q13.1
Amino acid length	300	330	346
% amino acid identity	33% to FFA2	33% to FFA1	34% to FFA1
	34% to FFA3	43% to FFA3	43% to FFA2
Activating fatty acid (carbon chain length)	C6–C22, saturated and unsaturated	C1–C6	C1–C6
Synthetic agonists (commercially available)	Rosiglitazone; troglitazone; GW9508X		
G protein coupling	$G\alpha_{q/11}$ and $G\alpha_{i/o}$	$G\alpha_{i/o}$ and $G\alpha_{q/11}$	$G\alpha_{i/o}$
Human tissue distribution	Highest expression in pancreatic islets, also in gastrointestinal tract, brain, and monocytes	Immune cells including peripheral blood leukocytes, neutrophils, eosinophils; adipocytes; distal ileum; colon	Adipose tissue; spleen; lymph node; bone marrow; peripheral blood mononuclear cells
Tissue function	Potentiation of GSIS in pancreatic islets; increase in glucagon secretion	Stimulation of adipogenesis; inhibition of lipolysis; activation of polymorphonuclear cells	Increase in leptin production
Disease	Type 2 diabetes		
Knockout animal	Yes	Yes	No
Phenotype of knockout	Reduction in capacity of FFAs to augment GSIS	Loss of acetate-mediated reduction in plasma FFA levels	

5.71 ± 0.11 (Briscoe et al., 2003). Itoh et al. (2003) used a similar method to identify LCFAs as agonists at FFA1 with potencies similar to those described by Briscoe et al. (2003). They also showed that methyl linoleate, a noncarboxyl-containing compound related to linoleic acid (C18:2), was unable to activate the receptor, indicating that a carboxyl group was required for agonist function (Itoh et al., 2003). Finally, while screening a subset of orphan receptors related to the leukotriene B_4 receptor, Kotarsky et al. (2003) also identified fatty acids as agonists for FFA1, although they found LCFAs to be less potent in the aequorin reporter assay used than alternative high throughput screens detailed by Briscoe et al. (2003) and Itoh et al. (2003). In this study Kotarsky et al. (2003) also first described non-LCFA agonists for FFA1; these were the insulin-sensitizing thiazolidinediones, rosiglitazone and MCC-555, and an experimental antiobesity compound, MEDICA 16. The role of thiazolidinediones and FFA1 in diabetes will be discussed in section VI.

The initial studies on FFA1 suggested that the receptor coupled to the Ca^{2+}-mobilizing G proteins, $G\alpha_q$ and $G\alpha_{11}$. In their study, Briscoe et al. (2003) found that FFA1 could activate a $G\alpha_{q/11}$- and $G\alpha_{i/o}$-dependent reporter gene assay without the need for exogenous chimeric or promiscuous G proteins. Furthermore, treatment with pertussis toxin did not affect FFA1 signaling, indicating that FFA1 was coupling solely to $G\alpha_{q/11}$ in this setting (Briscoe et al., 2003). Itoh et al. (2003) and Kotarsky et al. (2003) also concluded that FFA1 could couple to $G\alpha_{q/11}$ but both suggested that the receptor additionally coupled weakly to $G\alpha_{i/o}$. Studies on cells endogenously expressing FFA1 support $G\alpha_{q/11}$ coupling of the receptor (Fujiwara et al., 2005; Hardy et al., 2005). In particular, Shapiro et al. (2005) used the $G\alpha_{q/11}$ specific inhibitor, YM-254890 (Takasaki et al., 2004), on the pancreatic β-cell line, INS-1E, and this significantly re-

duced palmitic acid-induced mobilization of $[Ca^{2+}]_i$. YM-254890 has also been shown to inhibit the increase of glucose-stimulated insulin secretion (GSIS) induced by palmitic acid in isolated pancreatic islets (Latour et al., 2007). Support for the ability of FFA1 to couple weakly to $G\alpha_{i/o}$ comes from studies on the breast cancer cell line, MCF-7, in which treatment with pertussis toxin blocked the LCFA-induced elevation of $[Ca^{2+}]_i$ (Yonezawa et al., 2004). Thus, it seems likely that FFA1 G protein coupling is influenced by cellular background or assay endpoint.

B. Free Fatty Acid Receptors 2 and 3

At about the same time that LCFAs were reported to be agonists of FFA1, SCFAs with chain lengths of less than six carbons were described as the potential endogenous agonists for FFA2 and FFA3 (Brown et al., 2003; Le Poul et al., 2003; Nilsson et al., 2003). Brown et al. (2003) used a series of recombinant cell systems to confirm that FFA2 was activated by the carboxylic acid, acetate, and went on to show that the receptor was activated by a range of SCFAs with similar potency in $[Ca^{2+}]_i$ mobilization and $[^{35}S]GTP\gamma S$ binding assays. This result was confirmed by Le Poul et al. (2003) in both a Ca^{2+} mobilization-based assay system and a cAMP assay and also by Nilsson et al. (2003) who used both a reporter gene assay and a Ca^{2+} assay system. The rank order of potency was generally consistent between these different studies, with acetate (C2) and propionate (C3) being equipotent followed by butyrate (C4) and then valerate (C5) and formate (C1) (Brown et al., 2003; Le Poul et al., 2003; Nilsson et al., 2003). Because of the high sequence homology between FFA2 and FFA3, Brown et al. (2003) and Le Poul et al. (2003) also demonstrated that SCFAs were agonists at FFA3. The potencies of SCFAs at FFA3 were in a range similar to those at FFA2, although a different structure-activity
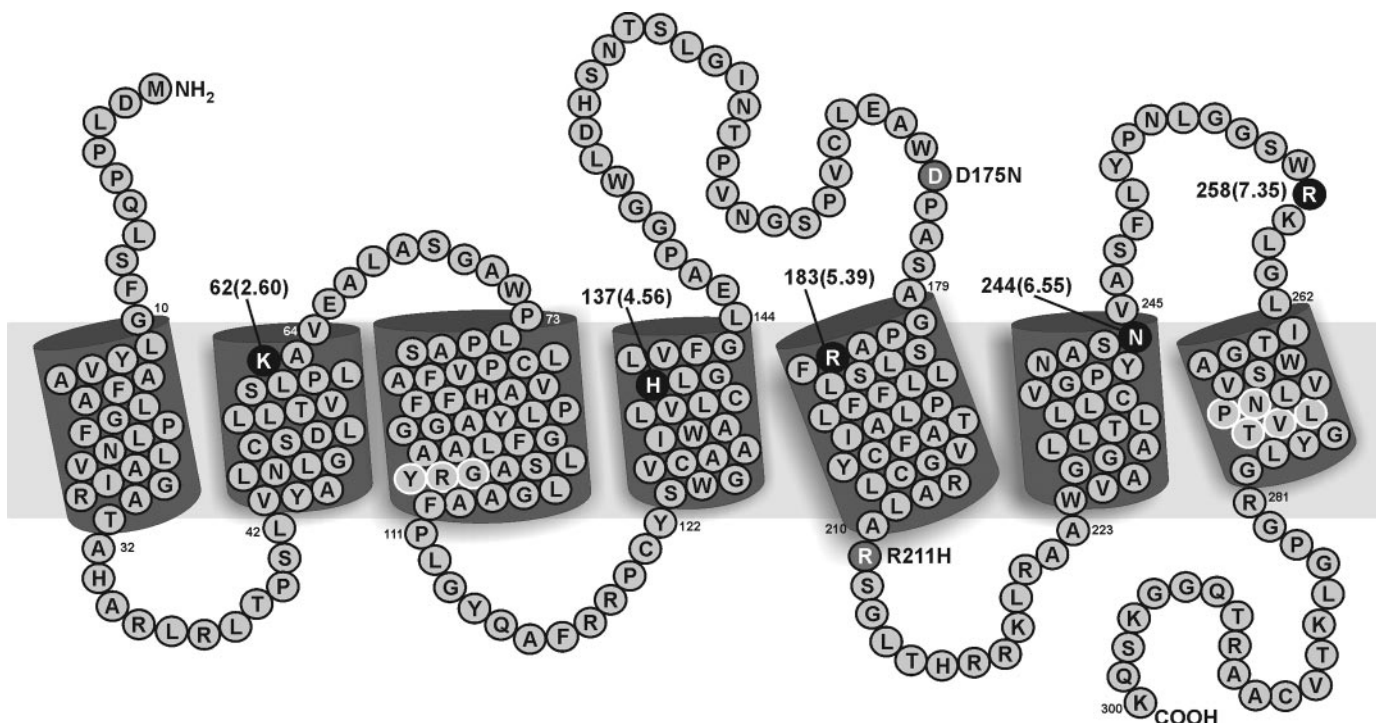
FIG. 1. Human FFA1 receptor. Snake plot of human FFA1 receptor (NP005924). TM helices were assigned according to the PHD algorithm (Rost, 1996). Conserved motifs common to class A GPCRs are shown in dark gray (white lettering) as are the two known human polymorphisms, Arg[211]His (Hamid et al., 2005; Ogawa et al., 2005) and Asp[175]Asn (Hamid et al., 2005). The FFA1 receptor has two putative N-glycosylation sites (Asn[155] and Asn[165]; motif: Asn-x-Ser/Thr) and two potential protein kinase C phosphorylation sites (Thr[215] and Ser[298], Ser/Thr-x-Arg/Lys). Basic residues conserved across the FFA receptor family and those additionally implicated in receptor activation by Tikhonova et al. (2007) are indicated in black (white lettering).

relationship was identified. Propionate, valerate, and butyrate were essentially equipotent at FFA3, with acetate and hexanoate showing measurably lower potencies (Brown et al., 2003; Le Poul et al., 2003). After heterologous expression of the human GPR42 sequence, no response to SCFAs could be detected (Brown et al., 2003). Similar to LCFAs at FFA1, SCFAs activate FFA2 and FFA3 with relatively low potency, generally in the mid-micromolar range.

Despite having the same repertoire of SCFA endogenous agonists and some overlapping tissue distribution, FFA2 and FFA3 have distinct G protein-coupling specificities. FFA2 has been shown to couple to both pertussis toxin-sensitive and -insensitive G proteins (Brown et al., 2003; Le Poul et al., 2003; Nilsson et al., 2003). However, Nilsson et al. (2003) concluded that the receptor coupled mainly through pertussis toxin-sensitive $G\alpha_{i/o}$ proteins as they observed a 70% reduction in the signal generated by FFA2 in a $[Ca^{2+}]_i$ mobilization assay in the presence of pertussis toxin. FFA3 couples efficiently to the pertussis toxin-sensitive G protein, $G\alpha_{o1}$, and agonist function can be measured in $[^{35}S]GTP\gamma S$ binding assays (Brown et al., 2003; Le Poul et al., 2003); it has also been shown that mouse FFA3 can reduce forskolin-induced cAMP production in CHO-K1 cells (Xiong et al., 2004). Meanwhile, Le Poul et al. (2003) found that it was necessary to coexpress FFA3 with the promiscuous G protein, $G\alpha_{16}$ (Milligan and Kostenis, 2006), to measure the

release of $[Ca^{2+}]_i$, suggesting an inability to couple to $G\alpha_{q/11}$. Neither FFA2 nor FFA3 has been tested for its ability to interact with a full panel of mammalian G proteins; however, FFA2 has been examined for its capacity to interact with a wide range of chimeric (Milligan and Rees, 1999) yeast-mammalian G protein α subunits, and it was found to interact with $G\alpha_{12}$, $G\alpha_{13}$, $G\alpha_{14}$, $G\alpha_{i1}$, and $G\alpha_{i3}$ (Brown et al., 2003) although the coupling of FFA2 to native $G\alpha_{12}$, $G\alpha_{13}$, or $G\alpha_{14}$ has not been confirmed.

Two further GPCRs have been shown to be activated by free fatty acids; GPR84 and GPR120 (Hirasawa et al., 2005; Wang et al., 2006). GPR84 was found to respond to medium chain fatty acids (C9–C14) (Wang et al., 2006), and GPR120 is activated by saturated (C14–C18) and unsaturated (C16–C22) LCFAs (Hirasawa et al., 2005). Neither receptor shares significant homology to FFA1, FFA2, or FFA3; therefore they will not be discussed further in this review.

V. Tissue Distribution of Free Fatty Acid Receptors

A. Tissue Distribution of Free Fatty Acid Receptor 1

The lack of specificity of the receptors for different fatty acids may indicate that selectivity of the activating fatty acid is determined by local tissue-specific environments and tissue-specific expression of each receptor. All three initial reports on FFA1 showed high levels of

receptor mRNA in the pancreas (Briscoe et al., 2003; Itoh et al., 2003; Kotarsky et al., 2003). Closer analysis of the distribution of expression of FFA1 mRNA in the pancreas showed levels enriched in islets and, in particular, the insulin-producing β-cells (Briscoe et al., 2003; Itoh et al., 2003). FFA1 expression has also been detailed in various pancreas-derived cell lines, including MIN6, β-TC-3, HIT-T15, and INS-1E (Briscoe et al., 2003; Itoh et al., 2003; Kotarsky et al., 2003; Shapiro et al., 2005). Tomita et al. (2005) further demonstrated expression of FFA1 in human islets and in islet cell tumors. Expression has also been detected in rat islets (Salehi et al., 2005; Feng et al., 2006). Such prominent β-cell expression was explained by a recent study on the promoter region of FFA1 that showed several highly conserved regions, one of which, HR2, is known to be a potent β-cell-specific enhancer of transcription (Bartoov-Shifman et al., 2007; Ridner et al., 2008). Although none of the initial articles on the expression pattern of FFA1 were able to detect expression of the receptor within the glucagon-producing α-cells of islets, a more recent report using a prospective FFA1 antibody showed the receptor to colocalize with glucagon, suggesting that the receptor can be found within α-cells (Flodgren et al., 2007).

FFA1 has been suggested to be ubiquitously expressed in all regions of the human brain with the highest expression in the substantia nigra and medulla oblongata (Briscoe et al., 2003). This suggestion was not supported, however, by a tissue distribution study performed by Kotarsky et al. (2003) using Northern blot analysis on human samples nor by Itoh et al. (2003), who were unable to detect FFA1 mRNA in rat brain. However, support for brain expression of FFA1 has come recently from the use of a putative FFA1-specific antibody in monkey brain, in which widespread expression was indicated (Ma et al., 2007). The expression of FFA1 was also detected in the subventricular and subgranular zones that form the neurogenic niche (Ma et al., 2007, 2008) and in the spinal cord and pituitary gland (Ma et al., 2007). Via Northern blot analysis of a variety of tissue samples, Kotarsky et al. (2003) detected expression of FFA1 in the human liver, heart, and skeletal muscle, but specific expression could not be seen in any of these areas in human or rat tissue by Briscoe et al. (2003) or by Itoh et al. (2003). FFA1 expression has also been reported in human immune cells, with the highest levels of mRNA detected in monocytes (Briscoe et al., 2003). In cultured cell lines, functional FFA1 has been detected in the human breast cancer cell lines, MCF-7 (Yonezawa et al., 2004) and MDA-MB-231 (Hardy et al., 2005). Expression of FFA1 has very recently been demonstrated within the enteroendocrine cells of mice (Edfalk et al., 2008).

B. Tissue Distribution of Free Fatty Acid Receptor 2

FFA2 mRNA can be detected in a variety of tissues, but the 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 detected in bone marrow and spleen but these are thought to reflect the expression of the receptor on immune cells (Le Poul et al., 2003). FFA2 expression on immune cells is not surprising because mouse FFA2 was first cloned from leukocytes (Senga et al., 2003). FFA2 has also been detected in neutrophils and eosinophils by hybridization to high-density oligonucleotide arrays (Nakajima et al., 2004). Nilsson et al. (2003) found expression of the receptor in skeletal muscle and heart, whereas FFA2 has also been reported in adipose tissue (Hong et al., 2005b; Ge et al., 2008), the breast cancer cell line, MCF-7 (Yonezawa et al., 2004), and rat distal ileum and colon (Karaki et al., 2006).

C. Tissue Distribution of Free Fatty Acid Receptor 3

FFA3 has a more widespread expression pattern than FFA2. Initial studies on FFA3 detected high levels of receptor mRNA in a range of normal human tissues with the highest levels of expression in adipose tissue (Brown et al., 2003). High levels of expression were also observed in the pancreas, spleen, lymph nodes, bone marrow, and peripheral blood mononuclear cells (Brown et al., 2003; Le Poul et al., 2003). FFA3 was detected in 3T3-L1 and 3T3-F442A preadipose cell lines, and although absolute levels remained low, expression was found to increase upon differentiation (Brown et al., 2003). The expression of FFA3 in adipose cells was confirmed by Xiong et al. (2004), who identified mRNA corresponding to FFA3 in human adipose tissue and mouse white adipose tissue and in differentiated cells from the mouse adipogenic cell line, Ob-Luc. However, adipose expression of FFA3 was brought into question by Hong et al. (2005b), who were unable to detect FFA3 expression in human adipose tissue, in cultured preadipocytes or adipocytes, or in 3T3-L1 cells, despite using the same probes for receptor mRNA (Hong et al., 2005b). The discrepancy of FFA3 expression in these studies is yet to be resolved. Finally, two recent patent applications from Arena Pharmaceuticals (Leonard and Hakak, 2005; Leonard et al., 2006) indicate that both FFA2 and FFA3 may also be expressed in pancreatic islet cells and that mRNA for both receptors is subject to up-regulation in *db/db* diabetic mice. These findings are yet to be published in the scientific literature and expression of FFA2 and FFA3 in the pancreas has yet to be confirmed independently.

VI. Free Fatty Acid Receptor 1 Synthetic Agonists and Antagonists

The expression of FFA1 in pancreatic β-cells has made it an attractive target for the development of small molecule agonists and antagonists. The first published re-

port of agonists at FFA1 was based on a 3-(4-{[*N*-alkyl]amino}phenyl)propanoic acid template (Garrido et al., 2006). Using a Gal4/Elk1/luciferase reporter assay these authors found that a range of these compounds had potencies in the nanomolar range compared with the micromolar potencies of LCFAs. They also reported that a carboxylic acid moiety was not essential for agonist action at the receptor; replacement of the carboxyl group with an amide group had no effect on potency, although the presence of such a feature resulted in more efficacious compounds. The same group also identified a FFA1-selective antagonist, GW1100 (Briscoe et al., 2006). A further set of agonists at FFA1 based on a 3-aryl-3-(4-phenoxy)-propionic acid template with significantly higher potency than any fatty acid have also been described (Song et al., 2007). High-throughput screening identified the templates for both of these sets of compounds. Conversely, information on the binding cavity of FFA1 allowed virtual screening to be used to identify novel synthetic agonists of the receptor (Tikhonova et al., 2008). This method also led to the identification of an antagonist that contained a nitro group in replacement of the carboxylate (Costanzi et al., 2008; Tikhonova et al., 2008). To date, no synthetic agonists or antagonists for FFA2 or FFA3 have been described.

In addition to the agonists and antagonists identified by high-throughput screening or virtual screening, two thiazolidinediones, rosiglitazone and troglitazone, have been shown to act as agonists at FFA1 (Kotarsky et al., 2003; Stoddart et al., 2007). When screening a subset of ligands of clinical interest using a FFA1-activation reporter gene assay, Kotarsky et al. (2003) demonstrated that rosiglitazone in the micromolar range could activate the receptor. The agonist action of rosiglitazone was confirmed in a [^{35}S]GTPγS binding assay developed for FFA1, and it was additionally found that the related compound troglitazone was a more potent activator of the receptor (Stoddart et al., 2007). It has recently been shown that rosiglitazone is likely to be a partial agonist at endogenously expressed FFA1 as it can inhibit palmitate-stimulated glucose release from pancreatic β-cells yet also acts as an agonist in the absence of palmitate (Meidute Abaraviciene et al., 2008). Both rosiglitazone and troglitazone are traditionally described as peroxisome proliferator-activated receptor γ (PPARγ) agonists. They are orally available insulin-sensitizing agents that act to reverse insulin resistance in target tissues, and rosiglitazone maleate (Avandia) is currently used in the treatment of type 2 diabetes. It is now clear that not all of the effects of thiazolidinediones can be explained by their action on PPARγ (Feinstein et al., 2005), indicating that activation of FFA1 by rosiglitazone and troglitazone may account for the growing number of described PPARγ-independent effects of these compounds. Recognizing the potential of thiazolidinedione actions through FFA1, Tan et al. (2008) recently screened a thiazolidinedione-based library for novel FFA1 ligands and

have developed several agonists for FFA1 that show no affinity for PPARγ (Tan et al., 2008).

VII. Molecular Basis of Free Fatty Acid Receptor 1 Activation

Despite clinical interest in the FFA receptor family and a growing knowledge of FFA1, FFA2, and FFA3 signaling pathways, little has been revealed about the molecular basis of ligand interaction until recently. This is due, in part, to the relatively recent deorphanization of the family and also to the fact that radioligand binding assays have been impossible to develop in the absence of high-affinity ligands. Instead, residues critical for ligand binding have been inferred from lost or impaired signaling of receptor mutants.

To date, FFA1 is the only member of the family to be subjected to a thorough analysis of its binding pocket. Using the 30 residues hypothesized by Surgand et al. (2006) to line the GPCR transmembrane binding cavity and additional iterative computational modeling, Tikhonova et al. (2007) identified four polar amino acids, three basic [His[137] (4.56), Arg[183] (5.39), and Arg[258] (7.35)] and a neutral [Asn[244] (6.55)] residue, that are likely to be involved directly in ligand:receptor interaction. Arg[183] (5.39), Asn[244] (6.55), and Arg[258] (7.35) are situated at the top of TMs 5, 6, and 7, respectively [indicated by their Ballesteros and Weinstein (1995) numbering and highlighted in Fig. 1] and were each shown to be involved in signal transduction, probably by anchoring the carboxylic head group of both linoleic acid (Sum et al., 2007) and the small molecule agonist, GW9508X (Tikhonova et al., 2007). Of these residues, Arg[183] (5.39) and Arg[258] (7.35) were required to achieve full potency and efficacy for linoleic acid but were essential for any signal generation via GW9508X (Sum et al., 2007).

A number of other residues in FFA1 were found to be involved in receptor activation in addition to the amino acids required to coordinate the carboxylate head group of the LCFAs. It is noteworthy that His[86] (3.32) and, in particular, His[137] (4.56), were found to make aromatic contacts with GW9508X (Tikhonova et al., 2007), although His[86] (3.32) makes stronger contacts with the full and partial agonists more recently described by Tikhonova et al. (2008). An additional hydrophilic interaction between Tyr[91] (3.37) and GW9508X, but not linoleic acid, is thought to account for the greater potency of the synthetic agonist than that of the endogenous ligands (Sum et al., 2007; Tikhonova et al., 2007).

The binding site model generated by Tikhonova et al. (2007) was very recently used by the same group to virtually screen 2.6 million "drug-like" compounds, resulting in the identification of 15 compounds acting at FFA1, incorporating full agonists, partial agonists, and pure antagonists (Tikhonova et al., 2008). A critical part of the virtual screening process involved definition of the

three-dimensional pharmacophore, which included clusters based on interactions with either Arg[183] (5.39), Asn[244] (6.55), and Arg[258] (7.35) or Tyr[91] (3.37) and His[137] (4.56). Crucially, the relationship between ligand class and amino acid interactions validates not only the binding pocket model but also the hypothesis that a carboxylate is important for agonist function: full agonists were more sensitive to mutation not only at both the critical interacting amino acids of Arg[183] (5.39) and Asn[244] (6.55) but also at His[137] (4.56) and His[86] (3.32). Conversely, a partial agonist was less sensitive to receptor mutation at these residues (Tikhonova et al., 2008); thus, the charged amino acids and Asn[244] (6.55) seem to be essential for high-affinity interactions with ligand and full receptor activation. Finally, as indicated earlier, the carboxylate was deemed to be important for receptor activation because the identified pure antagonist contained a nitro group instead of a carboxylate (Tikhonova et al., 2008). This finding is in agreement with results of other studies in which methyl substitutes of LCFAs have reduced activity or lack activity at FFA1 (Itoh et al., 2003; Salehi et al., 2005).

To date, there is only one mutagenic study of the remaining FFA receptors. In their initial deorphanization article, Brown et al. (2003) mutated each amino acid that differed between human FFA3 and its highly related pseudogene, GPR42. It is noteworthy that muta-tion of FFA3 Arg[174] (5.28) in extracellular loop 2 to its respective GPR42 residue, tryptophan, produced a version of FFA3 that was no longer able to respond to propionate. Conversely, introduction of Arg[174] into GPR42 rendered the previously unresponsive protein able to generate signals to propionate. Furthermore, a mutation at the bottom of TM6, Leu[223]Val (6.37), a region implicated in GPCR activation of G protein, caused FFA3 to display constitutive signaling. Although there are no studies to date on the binding pocket of FFA2 and FFA3, alignment of the three receptors (Fig. 2) indicates that His (4.56), Arg (5.39), and Arg (7.35) are conserved between FFA1, FFA2, and FFA3 and the polarity of Asn[244] (6.55) is retained in FFA2 and FFA3 [His (6.55)]. Thus, it seems reasonable to predict that the carboxylate of the SCFAs interacts with these receptors in a manner similar to that of FFA1.

VIII. Physiological Roles of Free Fatty Acid Receptors

A. Pancreatic Role of Free Fatty Acid Receptor 1

There is a strong link between obesity, type 2 diabetes, and circulating plasma levels of fatty acids; hence the endogenous expression of FFA1 in human pancreatic β-cells and in a range of transformed pancreatic β-cell lines has initiated a series of studies into the function of

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FFA2    --MLPD--WKSS---LILMAYIIIFLTGLPANLLALRAFVGRIRQPQPAPVHILLLSLTL  53
FFA3    MDTGPDQSYFSGNHWFVESVYLLTFLVGLPLNLLALVVFVGKLQR-RPVAVDVLLLNLTA  59
FFA1    MDLPPQ---------LSFGLYVAAFALGFPLNVLAIRGATAHARL-RLTPSLVYALNLGC  50
           *:          :  :  *: *  *:* *:**:   ..:  :  :..  :  *.*

FFA2    ADLLLLLLLPFKIIEAASNFRWYLPKVVCALTSFGFYSSIYCSTWLLAGISIERYLGVAF  113
FFA3    SDLLLLLFLPFRMVEAANGMHWPLPEILCPLSGFIFFTTIYLTALFLAAVSIERFLSVAH  119
FFA1    SDLLLTVSLPLKAVEALASGAWPLPASLCPVFAVAHFFPLYAGGGFLAALSAGRYLGAAF  110
         :****  :  **::  :**   .  *  **  :*.:  ..  .:  .:*    :**.:*  *:*..*.

FFA2    PVQYKLSRRPLYGVIAALVAWVMSFGHCTIVRIVQYLN---TTEQVRSGNEITCYEN---  167
FFA3    PLWYKTRPRLGQAGLVSVACWLLASAHCSVVYVIEFSG---DISHS-QGTNGTCYLE---  172
FFA1    PLGYQAFRRPCYSWGVCAAIWALVLQGLGLVEGLEAPGGWLDHSNTSLGINTPVNGSPVC  170
         *:  *:     *    .  ...* :     *   :*  ::  .      .:   *  :.   .

FFA2    FTDNQLDVVLPVRLELCLVLFFIPMAVTIFCYWRFVWIMLSQPLVGAQRR-RRAVQLAVV  226
FFA3    FRKDQLAILLPVRLEMAVVLFVVPLIITSYCYSRLVWILGRG---GSHRRQRRVAQLLAA  229
FFA1    LEAWDPASAGPAFFSLSLLLFFLPLAITAFCYVGCLRALARSG--LTHRRKLRAAWVAGG  228
         :    :        *.*::.::**.:*:  :*  :**     :  :      ::**  *.. :

FFA2    TLLNFLVCFGPYNVSHLVGYHQRKSP-WWRSLAVVFSSLNASLDPLLFYFSSS-------  278
FFA3    TLLNFLVCFGPYNVSHVVGYICGESP-AWRIYVTLLSTLNSCVDPFVYYFSSSGFQADFH  288
FFA1    ALLTLLLCVGPYNASNVASFLYPNLGGSWRKLGLITGAWSVVLNP----------------  273
         :**.:*:*.****.*::..:   :   **   :  .:  .  ::*

FFA2    -VVRRAFGRGLQVLRNQGSSLLGRRGKD------TAEGTNEDRGVGQGEGMPSSDFTTE   330
FFA3    ELLRRLCGLWGQWQQESSMELKEQGKGGEEQRADRPAERKTSEHSQGCGTGGQVACAES-  346
FFA1    -LVTGYLGRGPGLKTVCAARTQGGKSQK------------------------------   300
           ::     *        .       :. .
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FIG. 2. Amino acid sequence alignment of FFA1, FFA2, and FFA3. Amino acid sequences corresponding to human FFA1, FFA2, and FFA3 were aligned using the ClustalW2 algorithm. Transmembrane regions are boxed with a dashed line. Residues implicated in the ligand binding site of the FFA1 and the corresponding residues in FFA2 and FFA3 are boxed with a continuous line. Accession numbers are as follows: human FFA1, NP005294; human FFA2, NP005297; and human FFA3, NP005295.

the receptor in these cells. The role of fatty acids in pancreatic β-cells has been well documented, and they seem to have both short- and long-term effects. Pancreatic β-cells control insulin secretion that is stimulated by an increase in blood glucose concentrations. Glucose is taken up into the β-cells through the non–insulin-dependent glucose transporter, GLUT2, and metabolized to acetyl CoA. The resulting change in the ATP/ADP ratio causes the closure of ATP-sensitive K^+ channels, which in turn depolarizes the plasma membrane. Depolarization opens voltage-dependent Ca^{2+} channels, and the subsequent Ca^{2+} influx prompts insulin granule exocytosis and is considered to be the major trigger for GSIS [for a comprehensive review of LCFAs and GSIS, refer to Prentki (1996) and Nolan et al. (2006)].

Fatty acids have been shown to play an important role in normal β-cell function because an increase in blood free fatty acid concentration augments GSIS (Seyffert and Madison, 1967; Stein et al., 1997). Fatty acids seem to be required for basal insulin secretion because the absence of circulating fatty acids impairs GSIS, which can be reversed by replacement with exogenous fatty acids (Stein et al., 1996). In contrast to the augmentation of GSIS by short-term elevation of plasma fatty acid levels, chronic elevation of levels of fatty acids has been shown to impair insulin secretion (Prentki et al., 2002) and induce β-cell apoptosis (Lee et al., 1994; El Assaad et al., 2003).

The role of FFA1 within β-cells has been investigated in a variety of ways. Initial studies in MIN6 cells using siRNA to silence FFA1 expression demonstrated that fatty acids were unable to amplify insulin release after receptor knockdown (Itoh et al., 2003), an observation confirmed by Salehi et al. (2005). Likewise, FFA1 silencing with siRNA in INS-1E cells resulted in a significant reduction in insulin secretion in the presence of fatty acids and a rise in $[Ca^{2+}]_i$ could no longer be detected (Shapiro et al., 2005; Schnell et al., 2007). The mechanism of FFA1-mediated insulin secretion from rat β-cells has been suggested to involve a reduction in current through voltage-gated K^+ channels and an increase in cAMP-dependent protein kinase activity (Feng et al., 2006).

An important finding by Salehi et al. (2005) was the possible antagonist effects of 2-bromo palmitate (2BrP) on FFA1. They showed that 2BrP inhibited linoleic acid-mediated phosphatidylinositol hydrolysis in human embryonic kidney HEK293 cells transiently expressing mouse FFA1 and in MIN6 cells expressing the receptor endogenously. 2BrP also showed partial agonist properties by causing a slight activation of phosphatidylinositol hydrolysis, although no attempt at quantification was undertaken (Salehi et al., 2005). 2BrP is traditionally described as an inhibitor of carnitine palmitoyltransferase (CPT-1). CPT-1 inhibition leads to a build-up of long-chain acyl-CoA within the cytosol (De-Fronzo, 1997), which acts as an effector to enhance insulin release (Prentki and Corkey, 1996). The possible overlapping action of 2BrP at FFA1 and CPT-1 needs to be further investigated to determine the extent to which 2BrP-mediated GSIS inhibition can be attributed to FFA1 in pancreatic β-cells.

The most compelling evidence for a role of FFA1 in normal β-cell function and in the development of type 2 diabetes has come from the study of FFA1 knockout mouse models. FFA1 knockout mice seem to have normal glucose homeostasis, but islets isolated from such mice have been found to have a reduced capacity to augment GSIS on exposure to fatty acids (Steneberg et al., 2005; Latour et al., 2007; Tan et al., 2008). It seems that FFA1 mediates around half of the potentiating effects of fatty acids on insulin secretion, as there were some residual enhancing effects of fatty acid treatment in the absence of FFA1 (Latour et al., 2007). When fed a high-fat diet, wild-type mice become obese, resulting in elevated plasma fatty acid levels, insulin resistance, hyperinsulinemia, and glucose intolerance, in short, all of the factors associated with type 2 diabetes. In contrast, FFA1 knockout mice, although also obese, did not develop glucose intolerance and demonstrated less insulin resistance than their wild-type littermates (Steneberg et al., 2005). The effect of pancreas-specific overexpression of FFA1 on the development of type 2 diabetes was investigated in the same study. FFA1 was overexpressed under the control of the pancreatic Ipf1/PDX-1 promoter in transgenic mice. In contrast to the FFA1 knockout mice, those overexpressing FFA1 were glucose-intolerant and hypoinsulinemic when fed a normal diet and progressed rapidly to display overt diabetes (Steneberg et al., 2005). This finding indicated that FFA1 may mediate both the short- and long-term effects of fatty acids on β-cell function. In contrast, four subsequent studies using three independently generated FFA1 knockout mice showed that deletion of FFA1 did not protect against the effects of long-term fatty acid exposure (Latour et al., 2007; Tan et al., 2008, Lan et al., 2008, Kebede et al., 2008). Two of these studies showed that incubation of islets isolated from wild-type and $FFA1^{-/-}$ mice for 72 h with fatty acids significantly reduced GSIS and islet insulin content, indicating that lipotoxicity is not dependent upon activation of FFA1 (Latour et al., 2007; Tan et al., 2008). The protective effects of deletion of FFA1 in the development of insulin resistance seen in the initial $FFA1^{-/-}$ study were not observed by Lan et al. (2008). They found that $FFA1^{-/-}$ mice developed the same degree of insulin resistance as $FFA1^{+/+}$ mice (Lan et al., 2008). Likewise, the study performed by Kebede et al. (2008) showed that $FFA1^{-/-}$ mice developed insulin-resistance in response to a high-fat diet to an extent similar to that seen for wild-type mice but that FFA1 plays a role in GSIS after high-fat feeding (Kebede et al., 2008).

Dissection of the role of FFA1 in pancreatic β-cells in early studies was hampered by the lack of high-affinity specific agonists or antagonists. Thus, the recent publication of specific FFA1 agonists and antagonists has provided further insights into the role of the receptor in GSIS and β-cell function. Briscoe et al. (2006) demonstrated that GW9508X, one of the FFA1 agonists based on the 3-(4-{[N-alkyl]amino}phenyl)propanoic acid template developed by GlaxoSmithKline, is able to increase GSIS in MIN6 cells in a concentration-dependent manner, although not to the same levels as linoleic acid. Conversely, the FFA1-specific antagonist, GW1100, blocked enhancement of GSIS by GW9508X in MIN6 cells but only partially inhibited GSIS produced by linoleic acid (Briscoe et al., 2006). Using thiazolidinedione-based FFA1-specific agonists, Tan et al. (2008) demonstrated that activation of FFA1 in isolated islets enhances GSIS, whereas treatment of islets isolated from $FFA1^{-/-}$ mice with a FFA1 agonist did not increase GSIS (Tan et al., 2008). Unlike long-term exposure to fatty acids, incubation of isolated islets from either wild-type or $FFA1^{-/-}$ mice for 72 h with a FFA1-specific agonist did not affect GSIS. Although traditionally used as a model of insulin-dependent type 1 diabetes, treatment of neonatal rats with steptozotocin (STZ) can also be used as a model for type 2 diabetes because STZ-treated rats have reduced β-cell mass, and the remaining β-cells have a reduced ability to respond to glucose. In this model, treatment of the diabetic rats with a FFA1-specific agonist restored some of the ability of β-cells to release insulin in the presence of glucose (Tan et al., 2008). There is still considerable debate as to whether an agonist or antagonist at FFA1 would be of therapeutic value in the treatment of type 2 diabetes. The original report on $FFA1^{-/-}$ mice showed that they were protected from insulin resistance in response to a high-fat diet (Steneberg et al., 2005), which suggests that an FFA1 antagonist could prevent the development of type 2 diabetes. However, the combination of evidence from subsequent studies on $FFA1^{-/-}$ mice and the effects of the FFA1-specific agonists on isolated islets and neonatal STZ rats indicate that a FFA1 agonist may be of use in the treatment of type 2 diabetes because it could potentiate GSIS, rather than increase basal insulin secretion as with many current antidiabetes treatments.

There is a large body of literature on the effects of LCFAs on a variety of tissues, although the role of FFA1 in many of these tissues remains to be investigated. In addition to their role in β-cells, recent observations indicate that FFAs can also potentiate glucagon release from α-cells of the pancreas and that FFA1 may be involved (Bollheimer et al., 2004; Olofsson et al., 2004; Hong et al., 2005a). Flodgren et al. (2007) were able to detect FFA1 mRNA in a hamster glucagonoma cell line, In-R1-G9, and found that the receptor colocalized with glucagon in the periphery of mouse islets, indicating expression in α-cells. Furthermore, treatment of both In-R1-G9 cells and isolated mouse islets with linoleic acid increased glucose-stimulated glucagon release in a concentration-dependent manner. Knockdown of FFA1 expression significantly reduced the ability of linoleic acid to increase glucagon exocytosis and pretreatment of the cells with linoleic acid to potentially remove existing receptor from the surface of the cells rendered additional fatty acid unable to increase glucagon secretion (Flodgren et al., 2007). It has recently been shown that $FFA1^{-/-}$ mice secrete significantly less glucagon than wild-type mice in response to elevated plasma fatty acid levels (Lan et al., 2008), supporting a role of FFA1 in the release of glucagon from the α-cells. As noted earlier, the finding that FFA1 is expressed on pancreatic α-cells is in direct contrast to a number of other studies: using in situ hybridization, Briscoe et al. (2003) and Itoh et al. (2003) were both unable to detect FFA1 in the periphery of rat islets and FFA1 mRNA could not be detected in human glucagonoma tissue (Tomita et al., 2005). The reasons for these discrepancies are unclear and remain to be unraveled. Previous studies on mouse islets found that a release of $[Ca^{2+}]_i$ was required for fatty acid-stimulated glucagon release (Olofsson et al., 2004). The study by Flodgren et al. (2007) did not monitor fatty acid-mediated release of $[Ca^{2+}]_i$, but because FFA1-mediated $[Ca^{2+}]_i$ release has been observed in β-cells (Shapiro et al., 2005), it is possible that the release of $[Ca^{2+}]_i$ in α-cells may also be mediated though FFA1.

It has recently been shown that FFA1 is expressed within the gastrointestinal tract of mice (Edfalk et al., 2008). It was demonstrated that the receptor colocalized in cells that stained for a variety of gut hormones including glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), which both increase insulin secretion. In mice, plasma levels of GLP-1 and GIP increase in response to the ingestion of a high-fat diet. In mice in which the $FFA1$ gene was replaced by a $lacZ$ reporter gene, therefore essentially knocked out, levels of GLP-1 and GIP were significantly reduced in response to a high-fat diet, indicating a role for the receptor in the secretion of these hormones (Edfalk et al., 2008). Another GPCR that is activated by medium- to long-chain fatty acids, GPR120, has also been linked to GLP-1 secretion. Expression of GPR120 has been found in the intestinal endocrine cell line STC-1 and has been shown to mediate an increase in GLP-1 secretion in response to fatty acids (Hirasawa et al., 2005).

Three polymorphisms in the open reading frame of human FFA1 have been reported, Arg[211]His (Hamid et al., 2005; Ogawa et al., 2005), Asp[175]Asn (Hamid et al., 2005) and Gly[180]Ser (Vettor et al., 2008). Hamid et al. (2005) found that the allelic frequency of the Arg[211]His polymorphism was similar in normal subjects and patients with type 2 diabetes, and this finding has been confirmed in a recent study (Vettor et al., 2008). In a recombinant cell system, they found no difference in the

potency of eicosatrienoic acid for FFA1 Arg[211]His or FFA1 Asp[175]Asn but the Asp[175]Asn receptor showed a reduction in maximal efficacy of this agonist. However, a study on a small cohort of Japanese men indicated that the Arg[211]His polymorphism was linked to insulin secretory capacity as the authors found that serum insulin levels were higher in His/His homozygotes compared with Arg/Arg homozygotes (Ogawa et al., 2005). The Gly[180]Ser polymorphism has been described recently and was shown to be more common in obese subjects and linked to impaired insulin secretion in response to oral glucose (Vettor et al., 2008). The full pharmacological effects of these polymorphisms have yet to be studied in detail.

B. Other Potential Roles of Free Fatty Acid Receptor 1

Expression of FFA1 has been detected in breast cancer cell lines in two separate studies (Yonezawa et al., 2004; Hardy et al., 2005). Previous epidemiological studies have indicated that women in countries with a high-fat diet have a significantly higher risk of developing breast cancer than those in countries with a low-fat diet (Cohen et al., 1993; Rose, 1997). The increased risk associated with a high-fat diet has been confirmed in a number of studies using animal models and cultured breast cancer cells (reviewed in Cohen et al., 1993). Recent studies on the breast cancer cell line, MDA-MB-231, showed that oleic acid stimulated cell proliferation, but palmitic acid induced apoptosis (Hardy et al., 2000, 2003). Yonezawa et al. (2004) demonstrated that oleic acid and linoleic acid, both unsaturated fatty acids, were able to mediate a rapid, transient rise in $[Ca^{2+}]_i$ in the human breast cancer cell line, MCF-7, but palmitic acid and stearic acid, both saturated fatty acids, were unable to trigger a $[Ca^{2+}]_i$ transient. The effect of oleic acid was sensitive to pertussis toxin treatment (Yonezawa et al., 2004). Hardy et al. (2005) found similar results with MDA-MB-231 cells. To dissect the role of FFA1 in these cells, they reduced the levels of FFA1 by siRNA treatment and found that the proliferative effects of fatty acid ligands were reduced (Hardy et al., 2005). Thus, FFA1 may be involved in cell proliferation and its potential role in cancer progression warrants further investigation.

C. Potential Roles of Free Fatty Acid Receptors 2 and 3

The initial studies on FFA2 and FFA3 all indicated that FFA2 was expressed at high levels, and FFA3 to a lesser extent, in a variety of immune cells (Brown et al., 2003; Le Poul et al., 2003; Nilsson et al., 2003). Le Poul et al. (2003) demonstrated that treatment of polymorphonuclear cells with acetate or propionate induced a rise in $[Ca^{2+}]_i$ and subsequent chemotaxis. This result confirmed those of various other studies in which SCFAs mediated the activation of leukocytes by Ca^{2+} mobilization (Naccache et al., 1988; Nakao et al., 1992; Cavaglieri et al., 2003) and implicated FFA2 in the well known effects of SCFAs on leukocyte chemotaxis and

movement toward sites of bacterial infection. High concentrations of SCFAs have been reported in connection with severe anaerobic infections (Ladas et al., 1979), which therefore have the potential to cause activation of FFA2 and mediate the recruitment of leukocytes to the site of infection. The effect of eliminating the expression of FFA2 on the immune response was not investigated in the two current reports of $FFA2^{-/-}$ mice (Dass et al., 2007; Ge et al., 2008); therefore, the role of FFA2 in immune cells remains unclear.

Normal blood concentrations of SCFAs are in the range of 100 to 150 μM acetate, 4 to 5 μM propionate, and 1 to 3 μM butyrate (Cummings et al., 1987). However, increased levels of SCFAs are associated with some disease states, including bacterial infections and the genetic condition, propionic acidemia, which results in the accumulation of propionate in the blood (Feliz et al., 2003). Alcohol consumption has been shown to increase circulating acetate concentrations by 250% (Siler et al., 1999), and levels of acetate may reach those required to activate FFA2 significantly. In patients with alcoholic hepatitis and cirrhosis, it is common to find polymorphonuclear neutrophils at the site of disease (Bautista, 2002). The defective inflammatory response may be due to desensitization of the immune response through activation by excess acetate. However, this theory is speculative because to date no studies have been undertaken to examine the contribution that FFA2 makes to the defective immune response in these situations.

In addition to infection, large numbers of bacteria are present in the healthy colon where they serve to digest polysaccharides, oligosaccharides, and proteins in the gut, producing high concentrations of SCFAs in the process. In human colon, the total concentration of SCFAs reaches between 60 and 130 mM, of which acetate is the most prevalent, whereas butyrate and propionate are found in approximately similar concentrations, with a molar ratio of 60:20:20, respectively (Cummings et al., 1987). SCFAs are produced by anaerobic bacterial fermentation of undigested dietary carbohydrates and polysaccharides from fiber and have been proposed to play a key role in the maintenance of colonic homeostasis (Wong et al., 2006). Irritable bowel disease (IBD) is characterized by chronic and spontaneously relapsing inflammation of the gut. The precise etiology of disease progression is still unknown and is proposed to involve a number of factors including environmental, genetic, microbial, and immunological factors (Fiocchi, 1998). It has been suggested that the exacerbated inflammatory response in the intestine results from an inappropriate reaction to a luminal agent (Farrell and Peppercorn, 2002). During the development of IBD the luminal wall can be compromised, and this leads to further up-regulation of the immune response to the gut. Neutrophils are proposed to play a key role in the development of IBD and other inflammatory processes in the gut because they are recruited to the colon and undergo acti-

vation to produce reactive oxygen intermediates and chemokines (Fiocchi, 1998). The release of SCFAs from the gut into the bloodstream may act as an erroneous signal to the immune system via activation of FFA2. If activation of FFA2 is shown to play a role in the development of IBD, new therapeutic agents for treatment of this disease could be developed and, thus, studies are ongoing.

As indicated earlier, FFA2 mRNA has been detected in rat distal ileum and colon (Karaki et al., 2006). In a recent study Dass et al. (2007) attempted to uncover the role of FFA2 in intestinal motility. They confirmed that FFA2 mRNA was present in the rat gut, with the highest levels detected in the colon, so they investigated the contribution of FFA2 to the effects of fatty acids in the gut. It was found that SCFAs inhibit the electrical field stimulation-contraction of both rat and mouse distal colon in a concentration-dependent manner. The rank order of potency of the SCFAs corresponds to that seen for activation of FFA2 artificially expressed in HEK293 cells (Brown et al., 2003; Le Poul et al., 2003). To further investigate the contribution of FFA2 to this effect FFA2 knockout mice were studied; however, in the $FFA2^{-/-}$ mice the same rank order and degree of inhibitory effects were evoked by SCFAs (Dass et al., 2007), indicating that FFA2 is not likely to mediate inhibition of peristalsis. To date, there have been no reports of FFA3 being expressed in the gut. However, although expression of FFA3 was not investigated in this study, the rank order of potency of SCFAs at FFA3 in recombinant systems is different from that reported by Dass et al. (2007), suggesting that FFA3 is also not likely to mediate the effects of SCFAs on gut motility.

Leptin is known as a potent suppressor of appetite through its actions on the central nervous system (Cohen et al., 2001). Administration of sodium propionate to mice has been reported to increase circulating leptin levels (Xiong et al., 2004), and both FFA2 and FFA3 have been shown to be expressed in adipose tissue (Brown et al., 2003; Le Poul et al., 2003; Xiong et al., 2004; Hong et al., 2005b), the tissue responsible for production of leptin. As detailed in section V.C, there is some debate about the expression of FFA3 in adipocytes. Brown et al. (2003), Le Poul et al. (2003), and Xiong et al. (2004) were all able to detect FFA3 mRNA in adipose tissue. Xiong et al. (2004) undertook studies to determine the function of FFA3 in adipocytes and in the production of leptin. Using the Ob-Luc cell line, they demonstrated that addition of SCFAs to these cells increased leptin expression. They found a similar rank order of potency as observed previously in HEK293 cells artificially expressing FFA3 (Brown et al., 2003; Le Poul et al., 2003), with acetate being significantly less potent than propionate or butyrate. To examine the specific contribution of FFA3 to the production of leptin and because they were also able to detect FFA2 in these cells, they used siRNA treatment to knock down the expres-

sion of FFA3. FFA3 knockdown almost completely abolished the ability of propionate to induce leptin reporter expression and consequently leptin production. They concluded, therefore, that activation of FFA3 in adipocytes is coupled to the production of leptin (Xiong et al., 2004).

In contrast with the findings of Xiong et al. (2004), Hong et al. (2005b) came to a different conclusion as to the importance of FFA receptors in adipocytes. They were unable to detect FFA3 mRNA in any cell line or tissue tested and therefore concentrated on the role of FFA2 in the differentiation of adipocytes. Using 3T3-L1 adipocytes, they demonstrated that treatment of the cells with acetate or propionate increased FFA2 mRNA levels and oil red O staining, which indicates the accumulation of lipids within cells. Knockdown of FFA2 expression by siRNA treatment and subsequent exposure to SCFAs resulted in a reduction in oil red O staining compared with that for wild-type cells, indicative of a role for FFA2 in the differentiation of adipocytes. Treatment of 3T3-L1 cells with acetate or propionate also inhibited β-adrenoceptor-stimulated lipolysis in a concentration-dependent manner, and this lipolysis was abolished when FFA2 expression was reduced by siRNA treatment (Hong et al., 2005b). Subsequently, Ge et al. (2008) also investigated the ability of FFA2 to inhibit lipolysis and the effect of this inhibition on plasma free fatty acid levels. To confirm the role of FFA2 they generated a FFA2 knockout mouse model and found that neither acetate nor propionate was able to inhibit lipolysis in primary adipocytes isolated from the $FFA2^{-/-}$ mouse. Because activation of FFA2 seems to inhibit lipolysis, the effect that FFA2 activation had on plasma free fatty acid levels was monitored. It was found that 15 min after injection of acetate, plasma free fatty acid levels were significantly reduced, although levels returned to baseline within 60 min (Ge et al., 2008). These studies strongly support a role for FFA2 in inhibition of lipolysis in adipocytes (Hong et al., 2005b; Ge et al., 2008), although the discrepancies in the expression of FFA3 in these cells and its role in leptin production (Xiong et al., 2004) have yet to be explained. 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.

Two key articles were published while this article was being prepared for publication. Lee et al. (2008) described the first synthetic ligand for FFA2. The first study of the binding pocket of FFA2 or FFA3 was described by Stoddart et al. (2008).

IX. Conclusions

The low potency of SCFAs at FFA1, FFA2, and FFA3 led to suggestions that they may act as surrogate agonists rather than function as the endogenous agonists

for these receptors (Milligan et al., 2006). However, the physiological role of fatty acids and their relatively high concentrations in blood mean that a high-affinity receptor would always be active. It may be postulated that these receptors are only activated physiologically under specific situations in which levels of fatty acids are unnaturally high. The use of fatty acids at high concentrations can lead to a series of problems because of their inherent detergent properties and ability to form micelles that can disrupt cell membranes. The development of specific agonists and antagonists for FFA1 helps to address these issues because they can induce GSIS in pancreatic β-cells in a manner similar to that of fatty acids, and their effectiveness is abolished in islets isolated from $FFA1^{-/-}$ mice. The expression pattern of FFA1 in pancreatic β-cells and the clear role of LCFAs as endogenous agonists have attracted considerable interest in the potential of this receptor as a therapeutic target for the treatment of type 2 diabetes (Costanzi et al., 2008), a condition that is treated inadequately with current approaches. However, both variations in data emerging from FFA1 knockout mouse models and the recognition of detrimental effects of maintained elevation of LCFAs that may result from chronic activation of FFA1 mean that the best strategy remains uncertain. FFA2 and FFA3 are also attractive potential targets for conditions that range from IBD to appetite control. The development of selective high-affinity agonist and antagonist ligands for these receptors will assist greatly in defining their physiological roles and in determining their suitability as therapeutic targets.

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