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Intercellular Lipid Mediators and GPCR Drug Discovery

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Abstract

G-protein-coupled receptors (GPCR) are the largest superfamily of receptors responsible for signaling between cells and tissues, and because they play important physiological roles in homeostasis, they are major drug targets. New technologies have been developed for the identification of new ligands, new GPCR functions, and for drug discovery purposes. In particular, intercellular lipid mediators, such as, lysophosphatidic acid and sphingosine 1-phosphate have attracted much attention for drug discovery and this has resulted in the development of fingolimod (FTY-720) and AM095. The discovery of new intercellular lipid mediators and their GPCRs are discussed from the perspective of drug development. Lipid GPCRs for lysophospholipids, including lysophosphatidylserine, lysophosphatidylinositol, lysophosphatidylcholine, free fatty acids, fatty acid derivatives, and other lipid mediators are reviewed.

Key Words: Lipid mediator, GPCR, Lipid, Lysophospholipid, Fatty acid, Drug discovery

INTRODUCTION TO GPCRS AND NEW TECHNOLOGY

G-protein-coupled receptors

In humans and other multicellular organisms, communication systems connect cells and tissues. Endocrine, immune, and neuronal systems are representative communication methods (Im, 2002). Chemical messages, such as, hormones, autacoids, and neurotransmitters are released from cells and regulate target cells, which have receptors for first messengers. G-protein-coupled receptors (GPCR) are the largest superfamily of receptors for signaling molecules (ligands). Ligands may be amino acids, amine derivatives, peptides, proteins, lipid molecules, and even entities as small as Ca^{2+}, the proton, and the photon (Im, 2002).

GPCRs have seven transmembrane α-helix domains (Im, 2002), and thus, are sometimes called seven transmembrane receptors (7TM receptors), because G-protein independent signaling have been found for GPCR-activated intracellular signaling (Rajagopal *et al.*, 2010; Shukla *et al.*, 2011). The binding and recognition of first messengers or ligands by GPCRs result in receptor conformational changes (Im, 2002), which lead to G protein activation and the subsequent modulation of effector molecules, such as, adenylyl cyclase, phospholipase C and D, and ion channels (Fig. 1) (Im, 2002). Accordingly, the levels of second messengers of cAMP, IP_3, and Ca^{2+} are increased and/or decreased. Protein phosphorylation and dephosphorylation by kinases and phosphatases represent down-stream signaling cascades of second messengers. Furthermore, the phosphorylations of GPCRs by GRKs leads to the recruitment of β-arrestins, which results in desensitization, internalization (recycling), and G-protein-independent signaling (Rajagopal *et al.*, 2010; Shukla *et al.*, 2011). Representative signalings of GPCRs for lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are illustrated in Fig. 1, although each GPCR may initiate unique signals in different cell types.

The identification of mammalian GPCR sequence β2-aderenergic receptor in 1986 opened a new era in pharmacology, the ‘receptor-hunting period’ (Dixon *et al.*, 1986; Im, 2002). In 2012, Robert J. Lefkowitz and Brian Kobilka received the Nobel Prize in chemistry by their contribution to “studies of GPCRs”. Many receptor molecules for lipid mediators like platelet-activating factor, prostaglandins, leukotrienes, and cannabinoids, have been cloned and identified as GPCRs at the DNA level (Im, 2004). Sequencing of the human genome resulted in the identification of 865 human GPCR genes (Fredriksson *et al.*, 2003), and 367 GPCRs, excluding olfactory receptors, are considered functional receptors (Vassilatis *et al.*, 2003). Based on data supplied by the International Union of Basic and Clinical Pharmacology Committee on recep-

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tor nomenclature and drug classification (NC-IUPHAR), 354 GPCRs are now recognized as non-chemosensory GPCRs (Sharman *et al.*, 2011; Davenport *et al.*, 2013). Of these 92 are still classified as 'orphan receptors', because their natural ligands have not been identified (Sharman *et al.*, 2011; Davenport *et al.*, 2013; Southern *et al.*, 2013). GPCRs for intercellular lipid mediators have been identified and their numbers continue to increase along with the discoveries of new lipid mediators (Im, 2004, 2009).

New technologies for GPCR drug discovery

Because GPCRs play important physiological roles in homeostasis, they are major drug targets for drug discovery. About 40% of drugs on the market act on GPCRs as agonists or antagonists (Howard *et al.*, 2001). Drug discovery based on GPCRs is an attractive project for pharmaceutical companies and academic scientists alike. However, technical problems must be overcome (Im, 2002). The traditional techniques of radioligand binding and GTPγS binding have been used along with measurement of second messengers like cAMP, IP_3, and Ca^{2+} and the protein phosphorylation of MAPKs (Yoshida *et al.*, 2012; Zhang and Xie, 2012). When a ligand structure is known, radioligand binding is the best way to measuring affinities with candidate compounds, but information on whether a test compound is agonist or antagonist is not available and the radiomaterials are strictly regulated, which militate against this analysis method (Yin *et al.*, 2009; Zhang and Xie, 2012; Southern *et al.*, 2013).

A major obstacle to GPCR drug discovery is that each GPCR utilizes different sets of G proteins, such as, G_s, G_i, G_q, or G_{12}. Therefore, to analyze, promiscuous Gα16, or chimeric G proteins like Gqi were popularly adapted for GPCR drug discovery, like Ca^{2+} measurements by FLIPR (Molecular Devices), by many companies (Kostenis, 2004). Another way of overcoming this hurdle is to screen for β-arrestin recruitment, which is a later response of GPCR than Ca^{2+} response (Southern *et al.*, 2013). Many other high-throughput screening methods have been devised for GPCR activation analysis, such as, the use of frog melanophores as host cells (Lerner, 1994) or of artificial cell lines transfected with promoter/reporter genes, such as, luciferase or β-lactamase (Bresnick *et al.*, 2003). Because recent excellent review articles are available (Yoshida *et al.*, 2012; Zhang and Xie, 2012), a new technology named TGF-α shedding would be briefly introduced, which was applied for GPCR identification for a lipid mediator, lysophosphatidylserine (LPS) (Inoue *et al.*, 2012).

TGF-α shedding is a new technique for GPCR ligand screening, signaling, agonism and antagonism (Inoue *et al.*, 2012). It uses an engineered plasmid encoding alkaline phosphatase and a TGF-α domain (AP-TGF-α). For example, HEK293 cells, which express endogenous TACE (also known as ADAM-17), are transfected with a candidate or testing GPCR along with the engineered AP-TGF-α. When GPCR is activated, TACE will cut off AP domain from AP-TGF-α, and thus, measures of alkaline phosphatase activities in media (AP release) and in cells (remaining AP-TGF-α) gives infor-

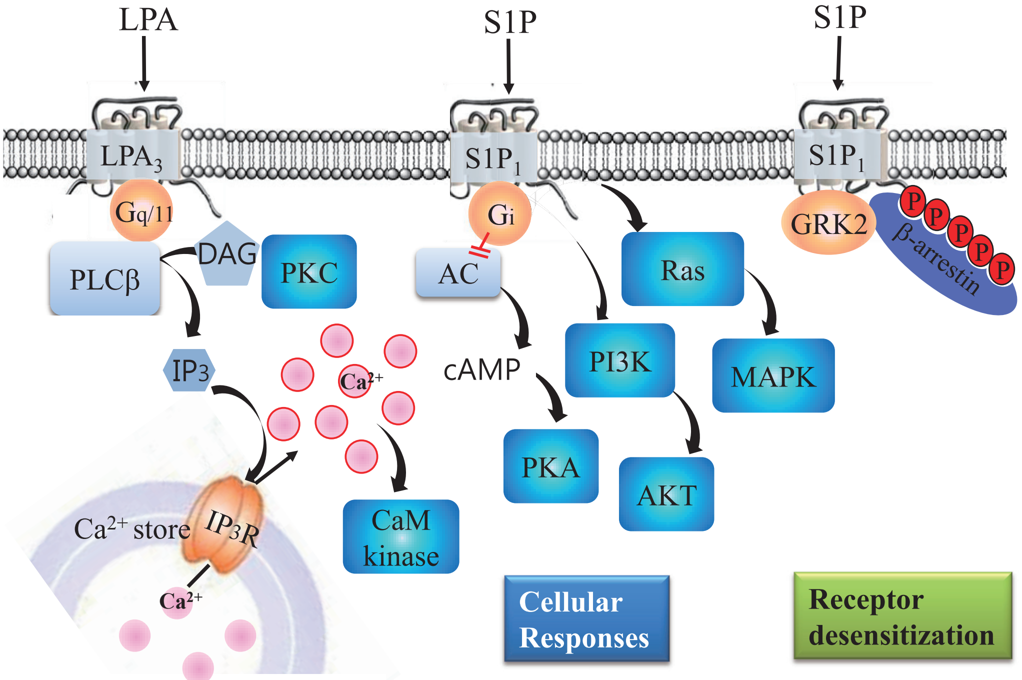

Fig. 1. Schematic diagram of lipid-mediated signal transduction pathways. For cellular responses, LPA activates LPA_3, which leads to activation of phospholipase C (PLC). Activated PLC produces IP_3 and diacylglycerol (DAG), DAG activates protein kinase C (PKC), and IP_3 mobilizes Ca^{2+} from internal Ca^{2+} stores by activating IP_3 receptors. The resulting increase in intracellular Ca^{2+} activates calmodulin-dependent protein kinases. Alternatively, S1P activates $S1P_1$, which leads to inhibition of adenylyl cyclase and the activations of ras, MAPK, PI3K, and AKT via Gi proteins. For desensitization, S1P activates $S1P_1$, which leads to GRK2 activation, $S1P_1$ phosphorylation, and to the recruitment β-arrestins by $S1P_1$. The recruited β-arrestins then inhibit $S1P_1$-G protein coupling.

mation on whether the activation state and degree of activation of GPCR (Inoue *et al.*, 2012). Of the 116 GPCRs tested, 104 were detected by the TGF-α-shedding assay, the highest percentage in a single assay format achieved for GPCR (Inoue *et al.*, 2012).

INTERCELLULAR LIPID MEDIATORS AND GPCRS

Intercellular Lipid Mediators

Intercellular lipid mediators are defined as chemical transmitters possessing water-insoluble moieties (Im, 2004). Intercellular lipid mediators are hormone-like signaling molecules, but they have the structural and physicochemical characters of lipids, such as, firt messengers of prostaglandins and leukotrienes (Im, 2004). These mediators can be divided into two groups: one with receptors in the plasma membrane and the other with intracellular nuclear receptors, such as, corticosteroids (Im, 2004). Many intercellular lipid mediators act on GPCRs in the plasma membrane, such as, cannabinoids, platelet-activating factor, prostaglandins, leukotrienes, and lysophospholipids (Im, 2004; Choi and Chun, 2013).

Certain lipid mediators have two types of receptors, that is, GPCR and nuclear receptors (Im, 2004). Estrogen and bile acids are representative examples, because they act not only through their nuclear receptors (estrogen receptor (ER) and farnesoid X receptor (FXR)) but also through GPCRs, GPR30 (now renamed GPER) for estrogen and TGR5 (now renamed GPBA) for bile acids (Prossnitz and Barton, 2009). Recently, GPR30 was found to be localized in endoplasmic reticulum (Arterburn *et al.*, 2009) and porcine GPR3 was observed in perinuclear membrane at the germinal vesicle stage of oocytes (Yang *et al.*, 2012). On the other hand, constitutive active rat GPR6 was shown to be localized in intracellular compartments (Padmanabhan *et al.*, 2009). Additionally, there is a growing evidence that GPCRs function in nuclear membranes (Zhu *et al.*, 2006; Goetzl, 2007). Therefore, it is incorrect to say that GPCRs act only in the plasma membrane. GPCR signaling in intracellular organelles, such as, endosomes and the nucleus, remains an interesting topic for future research (Irannejad *et al.*, 2013).

GPCRs for LPA and S1P

Since the first LPA receptor was identified in 1997, three Edg-subfamily members of LPA receptors and three purinergic (non-Edg) LPA receptors have been reported (Choi and Chun, 2013; Yanagida *et al.*, 2013). GPR23 and two additional purinergic GPCRs, that is, GPR92 and P2Y5, were confirmed as LPA receptors and renamed LPA$_4$, LPA$_5$, and LPA$_6$, respectively (Chun *et al.*, 2010; Yanagida *et al.*, 2013). Although GPR92/LPA$_5$ was reported to be a receptor for farnesyl pyrophosphate and N-arachidonylglycine (Oh *et al.*, 2008), their affinity was found to be lower than LPA in a β-arrestin assay and in a aequorin assay (Yin *et al.*, 2009). Peroxisome proliferation-activating receptor (PPARγ) has been reported to be a non-GPCR LPA receptor (McIntyre *et al.*, 2003), but further validation is required (Chun *et al.*, 2010). LPA$_1$ antagonist AM095, which is a lead compound for the treatment of idiopathic pulmonary fibrosis, systemic sclerosis, and scleroderma, is currently undergoing under clinical trials (Castelino *et al.*, 2011; Swaney *et al.*, 2011).

In addition, to the six LPA receptors, three orphan GPCRs have been proposed to be LPA receptors. GPR87 was reported to be an LPA receptor (Tabata *et al.*, 2007). P2Y10 has been reported to be a GPCR for S1P and LPA (Murakami *et al.*, 2008). P2Y10 was recently proposed to be an LPS receptor (Inoue *et al.*, 2012). GPR35 was also suggested to be an LPA receptor, especially for 2-arachidonyl LPA (Oka *et al.*, 2010). However, a consensus was reached in the scientific community to exclude these putative LPA receptors from the current list of LPA receptors, pending future experimental validation (Chun *et al.*, 2010).

Five members of the Edg-subfamily GPCRs (S1P$_{1-5}$) have also been reported to be S1P receptors (Chun *et al.*, 2010). Drug research on S1P receptor has been boosted by the finding of the S1P modulation of lymphocyte egress by FTY720 (Cyster and Schwab, 2012). FTY720 is an immune modulator and is phosphorylated *in vivo*. In the phosphorylated state, FTY720 acts as an agonist on four S1P receptors and as a functional antagonist on S1P$_1$ (Im, 2003). FTY-720 (fingolimod, Gilenya™; Novartis) was approved as a first-line oral treatment for relapsing-remitting multiple sclerosis by the US FDA (Obinata and Hla, 2012). Recently, a potent and selective S1P$_1$ antagonist, NIBR-0213, was shown to suppress autoimmune inflammation in an experimental autoimmune encephalomyelitis model in mice (Quancard *et al.*, 2012).

GPR3, GPR6, and GPR12 have been reported to be GPCRs for S1P and SPC. S1P was reported as a ligand for GPR3 and GPR6 and SPC for GPR12 (Im, 2004). These three GPCRs are phylogenetically similar to other GPCRs for lipid mediators, such as, LPA, S1P, and cannabinoids (Fig. 2) (Im, 2004), and were initially reported as adenylyl cyclase-activating orphan GPCRs (Eggerickx *et al.*, 1995). The constitutive activation of adenylyl cyclase by GPCR was continuously observed in GPR3-, GPR6-, and GPR12-expressing cells, not only in over-expressing cells *in vitro* but also in cells *in vivo*

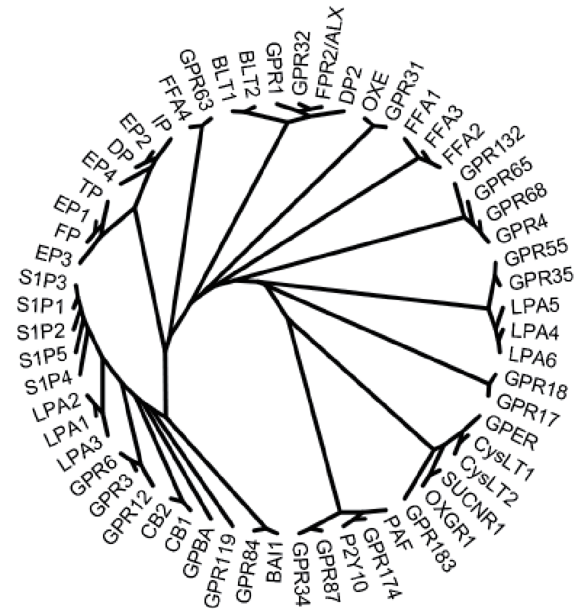

Fig. 2. Phylogenetic tree of lipid GPCRs. The phylogenetic tree was constructed using the Clustal Omega multiple sequence alignment and TreeIllustrator programs.

(DiLuigi *et al*., 2008b; Padmanabhan *et al*., 2009; Zhang *et al*., 2012). Furthermore, some reports support the activation of GPR3 by S1P. More specifically, the constitutive activation of adenylyl cyclase by GPR3 and the further activation of adenylyl cyclase by S1P, resulted in the accumulation of cAMP (Hinckley *et al*., 2005; Zhang *et al*., 2012). In addition, S1P was observed to induce the internalization of GFP-tagged porcine GPR3 (Zhang *et al*., 2012), whereas SPC did not (Yang

Table 1. Summary of recent GPCRs for intercellular lipid mediators

GPCR	Suggested Ligand	IUPHAR name	Remark	Ref
EDG2, EDG4, EDG7	LPA	LPA$_1$, LPA$_2$, LPA$_3$		Chun *et al*., 2010
GPR23, GPR92, P2Y5	LPA	LPA$_4$, LPA$_5$, LPA$_6$		Chun *et al*., 2010
EDG1, EDG5, EDG3, EDG6, EDG8	S1P	S1P$_1$, S1P$_2$, S1P$_3$, S1P$_4$, S1P$_5$		Chun *et al*., 2010
GPR3, GPR6, GPR12	S1P, SPC (?)		Constitutive activity	Ignatov *et al*., 2003; Uhlenbrock *et al*., 2002
GPR87	LPA (?)			Tabata *et al*., 2007
GPR35	LPA		2-arachidonyl LPA	Oka *et al*., 2010
GPR55	LPI		2-arachidonyl LPI	Oka *et al*., 2009
P2Y10	S1P, LPA (?) LPS (?)			Murakami *et al*., 2008 Inoue *et al*., 2012
GPR34	LPS		2-acyl LPS	Kitamura *et al*., 2012; Sugo *et al*., 2006
GPR174	LPS (?)			Inoue *et al*., 2012
GPR40, GPR43, GPR41, GPR120	Free fatty acids	FFA$_1$, FFA$_2$, FFA$_3$, FFA$_4$		Davenport *et al*., 2013
GPR84	Free fatty acids		Hydroxy fatty acids	Suzuki *et al*., 2013; Wang *et al*., 2006b
GPR119	OEA, LPC		Fatty acid derivatives	Overton *et al*., 2006; Soga *et al*., 2005
G2A	LPS, LPC, H$^+$, 9-HODE		Fatty acid derivatives (?)	Frasch *et al*., 2013; Obinata *et al*., 2005; Parks *et al*., 2005
GPR30	Estrogen	GPER		Prossnitz and Barton, 2009
TGR5/BG37	Bile acid	GPBA		Kawamata *et al*., 2003
TG1019	5-ox-ETE	OXE	oxo ETE	Grant *et al*., 2009
GPR31	12-S-HETE		Hydroxy ETE	Guo *et al*., 2011
BAI1	PS			Park *et al*., 2007
GPR17	CysLT (?) nucleotides (?)		Constitutive activity CysLTD$_4$, UDP-glucose	Ciana *et al*., 2006; Maekawa *et al*., 2009; Qi *et al*., 2013
GPR18	NAG (?)		Constitutive activity	Kohno *et al*., 2006
GPR183	Oxysterols		7α,25-dihydrocholesterol Constitutive activity	Hannedouche *et al*., 2011; Liu *et al*., 2011

S1P: sphingosine 1-phosphate; SPC: sphingosylphosphorylcholine; LPA: lysophosphatidic acid; FFA: free fatty acid; OEA: oleoylethanolamide; 5-oxo-ETE: 5-oxo-6E,8Z,11Z,14Z-eicosatetraenoic acid; LPC: lysophosphatidylcholine; LPI: lysophosphatidylinositol; LPS: lysophosphatidylserine; PS: phosphatidylserine; NAG: *N*-arachidonylglycine; 9-HODE: 9-hydroxyoctadecadienoic acid; 12-*S*-HEPE: 12-(*S*)-hydroxyeicosatetraenoic acid.

et al., 2012), and in the same study, SPC significantly delayed germinal vesicle breakdown in porcine oocytes. On the other hand, other did not observe any additional effect of S1P on the accumulation of cAMP, despite reproducing the increased production of cAMP in GPR3-expressing cells (Valverde *et al.*, 2009). Furthermore, S1P was not found to be a GPCR ligand in a β-arrestin PathHunter™ assay (Yin *et al.*, 2009; Southern *et al.*, 2013). Therefore, NC-IUPHAR is currently classified them as constitutively active orphan GPCR (Davenport *et al.*, 2013).

Nevertheless, there is growing evidence that GPR3 participates in the regulations of oocyte maturation and neurologic states. The constitutive activation of Gs proteins by GPR3 and its role in the prophase I meiotic arrest of oocytes have been shown in *Xenopus*, rodent, and in human oocytes and even in GPR3-knock-out mice (Freudzon *et al.*, 2005; Hinckley *et al.*, 2005; DiLuigi *et al.*, 2008a). Additionally, GPR3 has been proposed to participate in neurite outgrowth (Tanaka *et al.*, 2007), postnatal cerebellar development (Tanaka *et al.*, 2009), emotional-like responses (Valverde *et al.*, 2009), Alzheimer's disease (Thathiah *et al.*, 2009), the early phases of cocaine reinforcement (Tourino *et al.*, 2012), and neuropathic pain therapy (Ruiz-Medina *et al.*, 2011).

GPR63 was initially reported as a GPCR recognizing S1P and dioleoyl phosphatidic acid (Niedernberg *et al.*, 2003), but not much progress has been made on this pairing. Like the suggestion based on phylogenetic bioinformatic analysis of GPCRs, lipid ligands might not be correct for GPR63, because sequentially related neighbor GPCRs form a large class of peptide receptors (Vassilatis *et al.*, 2003; Im, 2004).

GPCRs for LPC and LPS

OGR1 (GPR68), GPR4, TDAG8 (GPR65), and G2A (GPR-132), which compose a subfamily of GPCRs, were initially reported to be lipid receptors for sphingosylphosphorylcholine (SPC), lysophosphatidylcholine (LPC), and psychosine (Im, 2005; Tomura *et al.*, 2005). Later, all four members were found to be proton-sensing GPCRs (Tomura *et al.*, 2005), and original reports on OGR1, GPR4, and G2A were later retracted. Although NC-IUPHAR has classified all four as orphan GPCRs (Davenport *et al.*, 2013), OGR1, GPR4, and TDAG8 continue to be reported as proton-sensing GPCRs in terms of the physiological and pathological relevances even in knockout mice (Okajima, 2013). On the other hand, G2A, a weak proton-sensing GPCR (Radu *et al.*, 2005), has been studied in various contexts.

Firstly, 9-hydroxyoctadecadienoic acid (9-HODE) was reported to be a ligand for human G2A/GPR132, but not for mouse G2A (Obinata *et al.*, 2005; Obinata and Izumi, 2009) and this was confirmed using the β-arrestin PathHunter™ assay (Yin *et al.*, 2009). Additionally, oxidized free fatty acids, such as, 9(*S*)-HODE, 9-hydroperoxyoctadecadienoic acid, and 11-HEPE, have been reported to be potent agonists of G2A, but LPC, 13(*S*)-HODE, and lauric acid were found to leave G2A unaffected in the β-arrestin assay (Yin *et al.*, 2009). Secondly, the regulatory functions of G2A have been studied in atherosclerosis, autoimmunity, and gallstone formation in G2A knockout mice (Johnson *et al.*, 2008; Kabarowski, 2009). Thirdly, Brantton's group suggested interesting roles for LPS in resolution of inflammation (Frasch and Bratton, 2012; Frasch *et al.*, 2013). LPS is made in activated neutrophils and LPS in the plasma membrane of neutrophils regulates macrophage efferocytosis and phenotype changes to M2 in a G2A-dependent manner in macrophages, and this finding was supported using G2A knockout mice (Frasch *et al.*, 2013). Previously, this group reported that LPC, LPS, and LPE mobilize neutrophil secretary vesicles and induce redundant signaling through G2A (Frasch *et al.*, 2007). A similar LPC-induced surface redistribution of G2A was shown in a previous study (Wang *et al.*, 2005). However, G2A involvement in lysophospholipid-induced Ca^{2+} signaling has been demonstrated only with antibody to G2A, such as, in experimental sepsis (Yan *et al.*, 2004; Frasch *et al.*, 2007).

GPR119 is largely restricted to pancreatic insulin-producing β cells and intestinal glucagon-like peptide-1-producing L-cells, and was first reported as a receptor for oleoyl-LPC, which enhances glucose-induced insulin secretion (Soga *et al.*, 2005). The ranking order lysophospholipid was found to be 18:1-LPC, 16:0-LPC>18:0-LPC>LPE, and LPI in RH7777 rat hepatoma cells stably expressing human GPR119 (Soga *et al.*, 2005). Later, *N*-oleoylethanolamide (OEA) was shown to activate GPR119 more potently than LPC (Overton *et al.*, 2006). *N*-Oleoyldopamine was also reported as an agonist of GPR119 (Chu *et al.*, 2010), and 2-oleoylglycerol was proposed as an endogenous agonist in food for GPR119 (Hansen *et al.*, 2011). Therefore, the stimulatory effect of dietary fat on incretin secretion (GLP-1 from L cells) and insulin secretion (β cells) may be mediated through GPR119 via multiple derivatives of oleate (C18:1) (Davenport *et al.*, 2013). Furthermore, the hypophagic effect (reduction of food intake) of GPR119 has made it a focus of anti-diabetic drug development (Overton *et al.*, 2008). In fact, over a hundred papers have described the development of potent GPR119 agonist and preclinical and clinical data suggest that GPR119 agonists will be the next generation of compounds used to treat type 2 diabetes mellitus (Shah and Kowalski, 2010; Davenport *et al.*, 2013).

LPS is an activator of mast cell degranulation, and has been reported to be a ligand for GPR34, which is highly expressed in mast cells (Sugo *et al.*, 2006); however, this pairing is somewhat controversial (Iwashita *et al.*, 2009; Liebscher *et al.*, 2011; Ritscher *et al.*, 2012). Kitamura *et al.* showed using several methods that GPR34 is a receptor of LPS with a fatty acid at the *sn*-2 position (Kitamura *et al.*, 2012). Recently, additional members of GPCRs, such as, GPR174 and P2Y10, were putatively suggested using a TGF-α shedding assay (Inoue *et al.*, 2012).

GPCRs for LPI

In addition to the classical cannabinoid receptors CB_1 and CB_2, cannabinoid GPCRs, have been implicated in studies on $CB_1^{-/-}$ and $CB_2^{-/-}$ knock-out mice and in studies using cannabinoid mimetic chemicals. Although phylogenetically distant from CB_1 and CB_2 receptors, several groups have reported that GPR55 is a cannabinoid receptor (Baker *et al.*, 2006; Johns *et al.*, 2007; Ryberg *et al.*, 2007). However, Sugiura *et al.* were unable to reproduce this result and instead suggested lysophosphatidylinositol (LPI, 2-arachidonyl-sn-glycero-3-phosphoinositol) as a ligand (Oka *et al.*, 2007, 2009), whereas other groups suggested that GPR55 is atypical as a cannabinoid receptor and in terms of its signaling (Johns *et al.*, 2007; Lauckner *et al.*, 2008). In 2010, NC-IUPHAR decided not to include GPR55 as a cannabinoid receptor (Pertwee *et al.*, 2010). However, the consensus is that LPI acts as an endogenous agonist on GPR55 (Pineiro and Falasca, 2012).

In this context, Yamashita *et al.* proposed that phylogenetically neighboring GPR55 and GPR35 evolved to share ligand recognition properties, that is, GPR35 recognizes 2-arachidonyl LPA and GPR55 recognizes 2-arachidonyl LPI (Yamashita *et al.*, 2013). GPR35 was initially reported to be a receptor for kynurenic acid, a metabolite of tryptophan (Wang *et al.*, 2006a). Although kynurenic acid is able to activate GPR35, it has considerably lower potency for human GPR35 than rat GPR35 (Jenkins *et al.*, 2011). Many surrogate ligands for GPR35, such as, zaprinast, cromolyn sodium, loop diuretics, and pamoic acid have been identified (Zhao and Abood, 2013). The importance of GPR35 in pain (spinal antinociception and inflammatory pain), heart disease, asthma, inflammatory bowel disease, and cancer has compelled scientists to find novel agonists and antagonists (Neetoo-Isseljee *et al.*, 2013).

GPCRS FOR FREE FATTY ACIDS

GPR40, GPR43, GPR41, and GPR120 act as receptors for short chain (GPR41 and GPR43), medium long chain (GPR40), and unsaturated long chain fatty acids (GPR120) (Talukdar *et al.*, 2011; Hara *et al.*, 2013). IUPHAR renamed them FFA_1 (GPR40), FFA_2 (GPR43), FFA_3 (GPR41), and FFA_4 (GPR120) (Davenport *et al.*, 2013). GPR84 has also been reported to act as a receptor for medium chain fatty acids of carbon chain lengths C9 to C14 (Wang *et al.*, 2006b). However, it has not been nominated yet to be FFA_5 (Davenport *et al.*, 2013). Interestingly, GPR84-deficient mice showed regulation of early IL-4 gene expression in activated T cells (Venkataraman and Kuo, 2005). In a recent study, it was suggested that medium-chain fatty acids with a hydroxyl group at the 2- or 3-position are more efficacious than non-hydroxylated fatty acids, and identified 6-*n*-octylaminouracil as a surrogate agonist (Suzuki *et al.*, 2013). Southern *et al.* confirmed that the medium chain fatty acids capric acid, undecanoic acid, and eicosatetraenoic acid evoke GPR84-mediated β-arrestin recruitment, cAMP, and calcium signaling (Southern *et al.*, 2013). Therefore, it may only be a matter of time before it is renamed FFA_5. The regulations of glucagon-like peptide-1 secretion from intestinal L cells and insulin from pancreatic β cells by free fatty acids via GPR120 and GPR40 are crucial considerations of the development of future treatments for diabetes. Because many review articles have been published on the topic of free fatty acids and their GPCRs, the reader is recommended to read (Talukdar *et al.*, 2011; Hara *et al.*, 2013).

CONSTITUTIVELY ACTIVE GPCRS, GPR17, GPR18, AND GPR183

GPR17 was initially reported to be a new dual uracil nucleotides/cysteinyl-leukotriens receptor (Ciana *et al.*, 2006). Later, Maekawa *et al*. suggested that GPR17 is a ligand-independent, constitutive negative regulator of $CysLT_1$ that suppresses $CysLT_1$-mediated function at the cell membrane (Maekawa *et al.*, 2009). Qi *et al.* recently confirmed this observation, that is, by lack of activation by UDP-glucose or CysLTs (Qi *et al.*, 2013).

Another group reported the differential expressions of two isoforms of GPR17 in human brain (short) and heart and lung (long). Furthermore, although activation of GPR17 by uracil nucleotides (UDP, UDP-glucose, and UDP-galatose) was observed, but not by cysteinyl-leukotrienes (LTD_4) (Benned-Jensen and Rosenkilde, 2010). Thus, the cognate ligands of GPR17 remain controversial (Davenport *et al.*, 2013). Because GPR17 has been reported to be a regulator of many physiological and pathological processes, including brain injury, spinal cord injury, oligodendrocyte differentiation, and food intake (Ren *et al.*, 2012; Coppi *et al.*, 2013; Franke *et al.*, 2013), it offers a good therapeutic target, especially for neurorepair after traumatic brain injury (Franke *et al.*, 2013). Both short and long forms of GPR17 have been reported to be constitutively activated via Gi protein activation (Benned-Jensen and Rosenkilde, 2010), and this constitutive activity could have functional meaning, as it does for GPR3.

GPR18, an orphan GPCR in lymphoid cell lines, such as, those of the spleen and thymus, was suggested to be a receptor for *N*-arachidonylglycine (NAG) (Kohno *et al.*, 2006). NAG is an endogenous metabolite of endocannabinoid anandamide (*N*-arachidonyl ethanolamine), and McHugh *et al.* found NAG and abnormal cannabidiol induced the cellular migration of BV-2 microglia, endogenously GPR18 expressing microglia, and exgenously GPR18-transfected HEK293 cells (McHugh *et al.*, 2010). Furthermore, GPR18 was found to be the most abundantly overexpressed orphan GPCR in 40 metastatic melanomas (Qin *et al.*, 2011). McHugh *et al.* reported that anandamide, ΔTHC, or NAG induced the migration of human endometrial HEC-1B cells, which express GPR18 (McHugh *et al.*, 2012). However, the pairing of GPR18 with NAG was not reproduced in a β-arrestin PathHunter^{TM} assay (Yin *et al.*, 2009; Southern *et al.*, 2013) or in GPR18-expressing neurons (Lu *et al.*, 2013), and GPR18 was found to be constitutively active to inhibit the apoptosis (Qin *et al.*, 2011).

EBI2 (also known as GPR183) was initially identified as one of nine up-regulated genes in Epstein-Barr virus (EBV)-infected Burkitt lymphoma cells and to show constitutive activity via Gi protein (Rosenkilde *et al.*, 2006). Oxysterols (oxygenated cholesterol derivatives) have been shown to activate EBI2, and 7α,25-dihydroxycholesteol was the most potent (Hannedouche *et al.*, 2011; Liu *et al.*, 2011), which thereby, unexpectedly linked EBI2 (an orphan GPCR that controls B-cell migration) and the immunological effects of certain oxysterols, and also suggested that the EBI2-oxysterol signaling pathway play an important role in the innate and adaptive immune systems (Spann and Glass, 2013).

GPCRS FOR OTHER LIPID MEDIATORS

Brain-specific angiogenesis inhibitor-1 (BAI1), an adhesion-type GPCR with an extended extracellular region, has been reported to be a phosphatidylserine (PS) recognition receptor (Park *et al.*, 2007). PS is known as a key “eat-me’ signal exposed on the outer leaflet of apoptotic cells, and BAI1 has been reported to function as an engulfment receptor for both the recognition and subsequent internalization of apoptotic cells (Park *et al.*, 2007; Bratton and Henson, 2008). The roles of BAI1 in the non-opsonic phagocytosis of Gram-negative bacteria, the fusion of healthy myoblasts, synaptogenesis, and the inhibition of tumor growth and angiogenesis via proteolytically processed extracellular domains, such as, vasculostatin (Vstat120), have been investigated (Kaur *et al.*, 2009; Cork and Van Meir, 2011; Das *et al.*, 2011; Duman *et al.*, 2013; Hochreiter-Hufford *et al.*, 2013).

Bile acids are being increasingly appreciated as complex metabolic integrators and signaling factors (Thomas *et al.*, 2008), although they have long been known to be essential in dietary lipid absorption and cholesterol catabolism (Watanabe *et al.*, 2006). TGR5 (now renamed GPBA) is a receptor for bile acids (Maruyama *et al.*, 2002; Kawamata *et al.*, 2003), and thus, bile acids signal not only through nuclear hormone receptors, such as, farnesoid X receptor α (FXR-α), but also through GPBA. The administration of bile acids to mice increased energy expenditure in brown adipose tissue, preventing obesity and resistance to insulin due to GPBA activation (Watanabe *et al.*, 2006). The targeted disruption of TGR5 in mice resulted in significant fat accumulation and body weight gain versus wild type mice when both were fed a high fat diet (Maruyama *et al.*, 2006). In another study, high TGR5 expression in gall bladder was observed with a marked reduction in gallstone development in TGR5$^{-/-}$ mice on a lithogenic diet (Vassileva *et al.*, 2006). The main indication for the development of TGR5 agonists is for the treatment of obesity, that is, to exploit the effect of the receptor on the regulating off energy expenditure (Fiorucci *et al.*, 2009).

GPR30 (now renamed as GPER) responds to estrogen with rapid cellular signaling (Prossnitz and Barton, 2009). A GPR30 antagonist, G-15, was discovered by high throughput flow cytometry (HyperCyt®) with fluorescent estrogen ligands (both cell permeable and non-permeable), which also elegantly showed GPR30 expression in endoplasmic reticulum and not in the plasma membrane (Arterburn *et al.*, 2009). GPER-selective ligands and GPR30 knockout mice have allowed the elucidation of GPER functions in many cases, which suggests that estrogen-mediated physiological responses may be mediated by either the receptor or a combination of GPER and nuclear ER receptors (Prossnitz and Barton, 2009). Furthermore, the physiological roles of GPER have expanded from reproductive, endocrine, immune and cardiovascular systems to nervous systems, as exemplified by studies on anxiolysis (Prossnitz and Barton, 2011; Tian *et al.*, 2013).

Serhan *et al.* discovered resolvin E1, resolvin D1, protectin, and maresin, which are all derivatives of omega-3 fatty acids, such as, DHA and EPA, and found they were anti-inflammatory and pro-resolving lipid mediators like lipoxin A_4, a pro-resolving mediator derived from arachidonic acid that plays important roles in the resolution of inflammation (Serhan *et al.*, 2002; Serhan *et al.*, 2008). In addition, resolvin E1 was found to activate GPR1/ChemR23 and to inhibit BLT_1. On the other hand, resolvin D1 and lipoxin A_4 activated GPR32 and FPR2/ALX for their pro-resolving responses (Serhan *et al.*, 2011). This topic has also been reviewed by experts (Serhan *et al.*, 2011).

OXE, formerly known as TG1019, was reported to recognize eicosatetraenoic acids and polyunsaturated fatty acids, including 5-oxo-6E, 8Z, 11Z, 14Z-eicosatetraenoic acid (5-oxo-ETE) (Hosoi *et al.*, 2002; Jones *et al.*, 2003; Brink *et al.*, 2004). 5-Oxo-ETE, the most potent agonist of OXE, is the most potent eosinophil chemotactic factor known (Hosoi *et al.*, 2002; Jones *et al.*, 2003). Because 5-oxo-ETE may be an important regulator of tissue infiltration and of the activations of eosinophils and neutrophils in diseases, such as, asthma, allergic rhinitis, arthritis, and psoriasis, OXE selective antagonists are currently under development as therapeutic agents for the treatment of asthma and other allergic diseases (Gore *et al.*, 2013; Powell and Rokach, 2013).

Furthermore, 12-(*S*)-hydroxyeicosatetraenoic acid (12-S-HETE), a 12-lipoxygenase metabolite of arachidonic acid has been suggested to be a high affinity ligand for GPR31, which is phylogenetically closest to OXE receptor (Guo *et al.*, 2011; Davenport *et al.*, 2013).

PERSPECTIVES OF INTERCELLULAR LIPID MEDIATORS AND THEIR GPCRS

Novel lipid mediators and GPCRs have been reviewed a number of times (Kostenis, 2004; Meyer zu Heringdorf and Jakobs, 2007; Grzelczyk and Gendaszewska-Darmach, 2013). In the present review, the statuses of GPCRs as intercellular lipid mediators are reviewed (Table 1). During the last decade, information on some GPCRs, such as, GPR3, GPR6, GPR12, and GPR63, in terms of ligand pairing has not progressed (Davenport *et al.*, 2013). The status of GPR23 (now renamed LPA_4) and the statuses of two additional members of purinergic LPA receptors, GPR92 (LPA_5) and P2Y5 (LPA_6) have been confirmed. Furthermore, GPR40, GPR43, and GPR41 are now confirmed GPCRs, and have been renamed free fatty acid receptors 1, 2, and 3 (FFA_1, FFA_2, and FFA_3, respectively) (Davenport *et al.*, 2013). In addition, GPR120 has been renamed fatty acid receptor 4 for long-chain, especially unsaturated fatty acids, like omega-3 DHA and EPA (FFA_4) (Davenport *et al.*, 2013). TG1019 has been confirmed as a receptor for 5-oxo-eicosatetraenoic acid (5-oxo-ETE) and renamed OXE receptor (Brink *et al.*, 2003). Drug development targeting TGR5 (GPR131, now renamed GPBA) and GPR30 (now renamed GPER) is being actively pursued for the treatment of lipid and glucose disorders (Davenport *et al.*, 2013). In addition, new GPCRs have been introduced, namely, GPR17, GPR18, GPR31, GPR32, GPR34, GPR35, GPR55, GPR84, GPR87, GPR119, GPR120, GPR174, GPR183, P2Y10, and BAI1 (Davenport *et al.*, 2013).

GPR17, GPR18, and GPR183 have been reported to be constitutively active like GPR3, GPR6, and GPR12 (Eggerickx *et al.*, 1995; Uhlenbrock *et al.*, 2002; Rosenkilde *et al.*, 2006; Benned-Jensen and Rosenkilde, 2010; Qin *et al.*, 2011), which might be a cause of that ligand-GPCR pairings have been controversial. Care must be taken when ligand-GPCR pairing is studied in overexpressed systems (Im, 2004), especially, if GPCR overexpression leads to constitutive activity, because this can alter ligand behavior (Kenakin, 2001). The constitutive activities of GPCRs, such as, GPR3, has physiological meaning via the constitutive activity and regulation of GPCR expression in specialized tissue areas (Freudzon *et al.*, 2005; Hinckley *et al.*, 2005; Tanaka *et al.*, 2007; Ruiz-Medina *et al.*, 2011). Therefore, investigations of constitutively active GPCR expression *in vivo* using knockout mice should be undertaken in addition to *in vitro* studies on suggested ligands and GPCR-overexpressing cells.

In studies on GPR87 and P2Y10, GPCR-Gα16 fusion was used as a tool to search endogenous ligands (Tabata *et al.*, 2007; Murakami *et al.*, 2008). Without confirmation by other assay systems or by other laboratories, the original suggested pairing with LPA and S1P could not be supported (Chun *et al.*, 2010; Davenport *et al.*, 2013). Although BAI1 and GPR31 were respectively reported in a single paper, multiple assays were carefully undertaken (Park *et al.*, 2007; Guo *et al.*, 2011). The pairings of P2Y10 and GPR174 with LPS were found in TGF-α shedding assay (Inoue *et al.*, 2012). Further studies are required to confirm the pairing results in the future.

Of the newly found lipid GPCRs, drug development targeting GPR119 is undoubtedly the most active field, because it is related to the treatment of diabetes (Shah and Kowalski, 2010; Ohishi and Yoshida, 2012). Fundamental studies on pathophysiologies of other GPCRs should provide bases for future GPCR drug development.

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