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# Novel Clusters of Receptors for Sphingosine-1-Phosphate, Sphingosylphosphorylcholine, and (Lyso)-Phosphatidic Acid: New Receptors for "Old" Ligands

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**Abstract** The (lyso)phospholipid mediators sphingosine-1-phosphate (S1P), lysophosphatidic acid (LPA), sphingosylphosphorylcholine (SPC), and phosphatidic acid (PA) regulate diverse cellular responses such as proliferation, survival and death, cytoskeletal rearrangements, cell motility, and differentiation among many others. Signaling is complex and many signaling events are mediated through the activation of cell surface seven transmembrane (7TM) G protein coupled receptors. Five high affinity receptors for S1P have been identified so far and named $S1P_{1,\ 2\ ,3\ ,4\ ,5}$ (formerly referred to as endothelial differentiation gene (edg)1, 5, 3, 6, 8). Recently, the orphan receptor GPR63 was identified a low affinity S1P receptor structurally distant from the $S1P_{1-5}$ family. The orphan GPR3, 6, 12 cluster, phylogenetically related to the edg and melanocortin receptors appears to be subject to modulation by S1P and SPC although all three receptors are strong constitutive stimulators of the Gαs-adenylyl cyclase (AC) pathway and would not require additional ligand stimulation but rather inverse agonism to control activity. Ovarian cancer G protein coupled receptor 1 (OGR1) and GPR4, two structurally closely related receptors were assigned in functional and binding studies as high affinity molecular targets for SPC. Very recently, however, both OGR1 and GPR4 were described as receptors endowed with the ability to signal cells in response to protons. LPA exerts its biological effects through the activation of G protein coupled $LPA_{1-3}$ receptors (formerly referred to as edg2, 4, 7). A fourth high affinity LPA receptor has been identified: P2Y9 (GPR23) structurally related to nucleotide receptors and phylogenetically quite distant from the high affinity $LPA_{1-3}$ cluster. This review attempts to give an overview about the existing families of lysophosholipid receptors and the spectrum of lipid agonists they use as high or low affinity ligands to relay extracellular signals into intracellular responses. Recently deorphaned lipid receptors, within and outside the known lipid receptor clusters will receive particular attention. J. Cell. Biochem. 92: 923–936, 2004. © 2004 Wiley-Liss, Inc.

**Key words:** GPCR; sphingosine-1-phosphate; lysophosphatidic acid; sphingosylphosphorycholine; phosphatidic acid; lipid receptor; lysosphingolipid mediator

## (LYSO)-PHOSPHOLIPIDS AND THEIR TARGET RECEPTORS

Sphingosine-1-phosphate (S1P), sphingosylphosphorylcholine (SPC), lysophosphatidylcholine (LPC), lysophosphatidic acid (LPA), and phosphatidic acid (PA) (Fig. 1) belong to a class of lipid signaling molecules appreciated to exert complex effects on target cells through activation of cell surface seven transmembrane (7TM) receptors or as intracellular second messengers [Pyne and Pyne, 2000a; Chun et al., 2002; Spiegel and Milstien, 2002]. Among those, S1P and LPA are the best studied lipid mediators. Interest in S1P has accelerated with the discovery that it is a ligand for the G protein

Abbreviations used: AC, adenylyl cyclase; doPA, dioleoyl phosphatidic acid; edg, endothelial differentiation gene; ERK, extracellular signal regulated kinase; GLD, globoid cell leukodystrophy; GPCR, G protein coupled receptor; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; MAPK, mitogen-activated protein kinase; OGR1, ovarian cancer G protein coupled receptor 1; PLC, phospholipase C; PTX, pertussis toxin; S1P, sphingosine-1-phosphate; SPC, sphingosylphoshorylcholine; SRE, serum response element; TDAG8, T cell death associated gene 8; 7TM, seven transmembrane.

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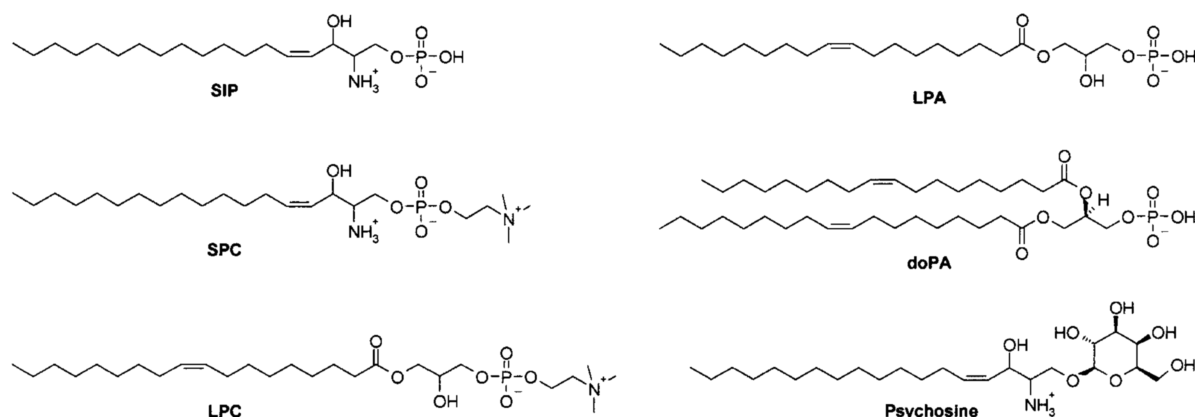


**Fig. 1.** Structures of selected (lyso)-phospholipids exerting effects via cell surface seven transmembrane G protein coupled receptors (7TM G protein coupled receptors (GPCRs)).

coupled receptor endothelial differentiation gene (edg)1 which was originally cloned as an early gene induced during the morphogenetic differentiation phase of angiogenesis [Hla and Maciag, 1990]. The discovery that LPA—originally known for its unglamorous role as an intermediate in intracellular lipid metabolism—signals through the 7TM edg2 receptor has similarly prompted a new era of LPA research dedicated to unravel LPAs multiple signaling pathways and (patho)-physiological actions [Hecht et al., 1996].

## High Affinity G Protein Coupled S1P and LPA Receptors and Phylogenetic Neighbors

The S1P-edg1 ligand–receptor pair rapidly triggered the identification of the related edg3, 5, 6, and 8 as high affinity receptors for S1P [for review see Spiegel and Milstien, 2003]. Similarly did the identification of edg2 as the first LPA receptor accelerate discovery of the remaining LPA receptors [Hecht et al., 1996]. The complete edg receptor family consists of eight members which can be divided into two subfamilies: edg1, 3, 5, 6, and 8 (now referred to as $S1P_{1,\ 3,\ 2,\ 4,\ 5}$) as high affinity S1P receptors and edg2, 4, and 7 (now referred to as $LPA_{1,\ 2,\ 3}$) with high affinity for LPA (Fig. 2). The high affinity LPA and S1P receptor genes are widely expressed in mammalian organ systems and their expression patterns are regulated throughout development. Their ubiquitous expression combined with the pleiotropic nature of their biological effects confer onto the lysophospholipid receptor cluster the ability to exert a plethora of physiological actions in the whole organism. S1P in particular is endowed with the ability to regulate cell migration [Wang et al., 1999], angiogenesis and vascular maturation [Lee et al., 1999; Liu et al., 2000; Garcia et al., 2001], cell survival, cardiac and central nervous system development [Kuppermann et al., 2000], and immunity [Brinkmann et al., 2002; Mandala, 2002]. LPA has been viewed initially as an intermediate in phospholipid biosynthesis but has emerged to an important bioactive lipid mediator with diverse biological activities ranging from smooth muscle contraction, platelet aggregation, alterations in blood pressure among many others to growth-factor like activities in almost every cell type. Given its activity on cell proliferation, migration, and survival and the link of aberrant LPA production and differential LPA receptor expression and signaling in certain types of cancer, it certainly belongs to the most pleiotropic molecules in the human body with roles in health and disease [Luquain et al., 2003; Mills and Moolenaar, 2003]. It is beyond the scope of this review, however, to highlight the emerging role of this bioactive lipid and its receptors in various (patho)-physiological settings. The non-edg receptors that are most similar to the edg receptors are the cannabinoid CB1 and 2 receptors followed by the family of melanocortin (MC) receptors as well as the small GPR3, 6, 12 family of rhodopsin-like Family A G protein coupled receptors (GPCRs) (Fig. 1). It should be emphasized that the CB1 receptor only displays about 30% amino acid homology with any edg protein. The $S1P_{1-5}$ receptor subfamily is about 35% identical to the $LPA_{1-3}$ receptor family and both groups are discriminated further by the presence of an intron in the region of the gene

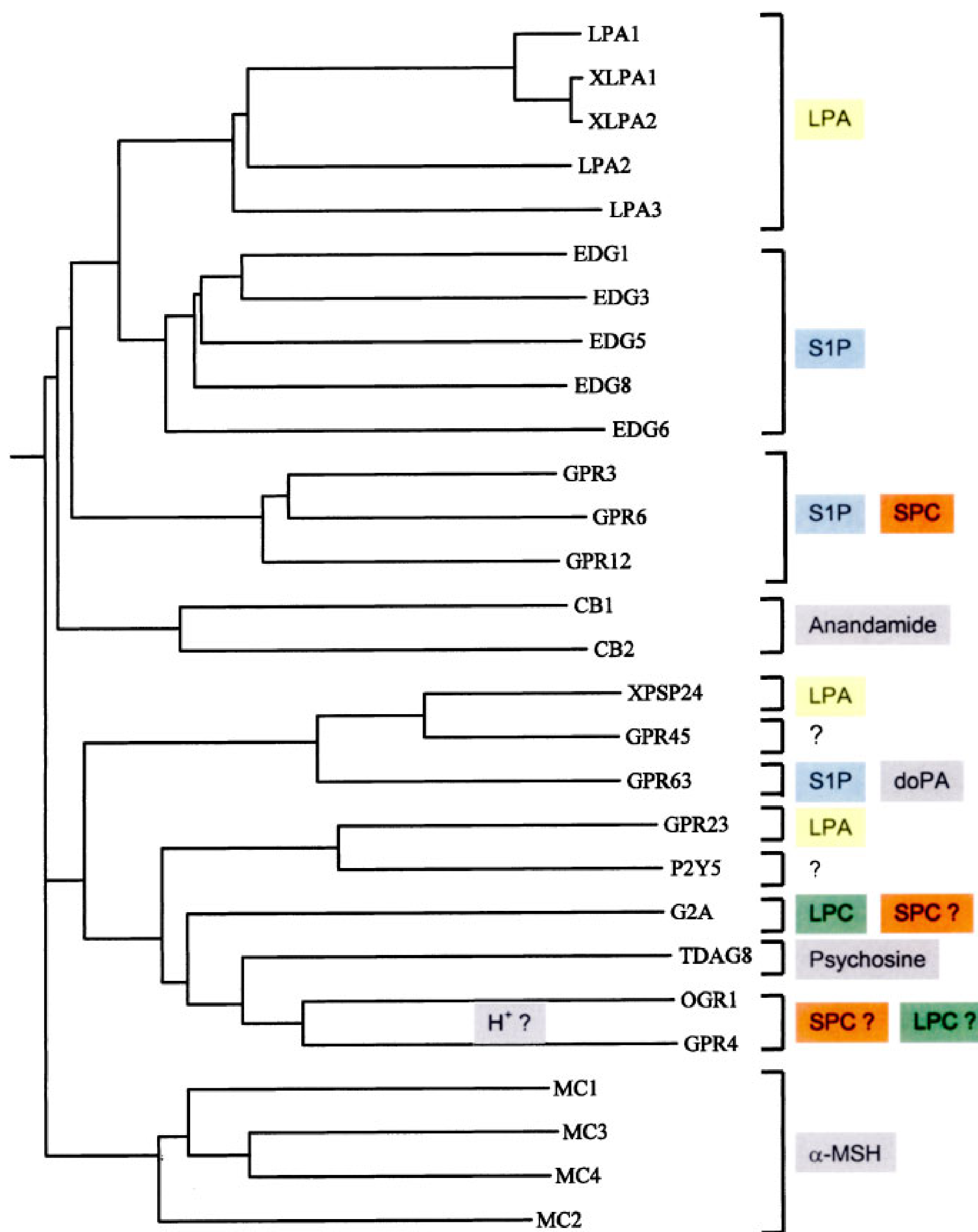


**Fig. 2.** Phylogenetic tree analysis of all high and low affinity sphingosine-1-phosphate (S1P), sphingosylphoshorylcholine (SPC), lysophosphatidic acid (LPA), and PA receptors as well as phylogenetically closely related receptors. Agonistic ligands reported for the various receptor classes are placed behind brackets. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

encoding the sixth transmembrane domain (TMVI) only in the $LPA_{1-3}$ group. The $S1P_{1-5}$ receptors are about 50% identical on the amino acid level, which implies that identification of subtype selective ligands will be challenging but not impossible.

## The Moderate Affinity S1P Receptor GPR63

In 1996, the first *Xenopus* LPA receptor (now referred to as xPSP24) was cloned using degenerate primers designed on the PAF receptor [Guo et al., 1996]. xPSP24 displays only 20%

amino acid identity to the S1P and LPA receptor family. Four years later, two mammalian homologs of xPSP24 were identified and named PSP24$\alpha$ and $\beta$ (now referred to as GPR45 and GPR63). GPR45 and 63 exhibit about 60% sequence identity to the xPSP24 receptor [Kawasawa et al., 2000a]. Both GPR45 and GPR63 were tested for their responsiveness to LPA in different functional assays, however, neither receptor mediated any effect in response to LPA [Kawasawa et al., 2000b]. In 2003, the hypothesis was tested that GPR63 may be activated by a physiologically active lipid distinct from LPA and in the course of these experiments S1P, dihydro-S1P (dhS1P), and dioleoyl phosphatidic acid (doPA) were identified as functional, albeit low affinity agonists [Niedernberg et al., 2003a]. Thus, the S1P family of receptors, so far comprising $S1P_{1-5}$ which exhibit high sequence homology to each other and bind S1P with high affinity [Pyne and Pyne, 2000a,b; Spiegel and Milstien, 2003] can be extended by an additional member with little sequence homology and moderate affinity for S1P, dhS1P, and doPA. Despite the high concentrations of S1P required to stimulate GPR63, it could play a role as (patho-)physiologically relevant S1P target since concentrations of S1P in plasma and serum are about 200 and 500 nM, respectively [Yatomi et al., 1997], and can even be higher upon autocrine or paracrine stimulation of cells [Hobson et al., 2001].

## High Affinity G Protein Coupled SPC and Related Receptors

As compared with the wealth of information available about S1P and LPA, little is known about SPC with respect to its molecular mechanism of action and physiological significance. It appears that many of the SPC effects are mediated by low affinity interaction with the $S1P_{1-5}$ receptor family [Meyer zu Heringdorf et al., 2002], however, the spectrum of biological activity of SPC is similar not identical to that of S1P [Orlati et al., 1998; van Brocklyn et al., 1999; Windh et al., 1999] and certain intracellular signaling events are exclusively mediated by an SPC receptor unresponsive to S1P [van Koppen et al., 1996]. SPC is a constituent of normal blood plasma and serum and various biological activities have been attributed to SPC such as cell migration, angiogenesis, wound healing, smooth muscle contraction, and cell proliferation among others, however, specific cell surface receptors have remained elusive until recently. The first clear functional evidence for a high affinity SPC receptor was obtained in experiments on atrial cardiomyocytes from guinea pigs: nanomolar concentrations of SPC activated the inwardly rectifying potassium channel $I_{K.Ach}$ only from the extracellular face of the plasma membrane in a pertussis toxin sensitive manner [Buenemann et al., 1996]. Interestingly, S1P was equally active in activating $I_{K.Ach}$ suggesting that both lipids are acting on the same receptor. However, no GPCR responding with equally high affinity to both S1P and SPC has been identified so far. Various S1P receptor subtypes do respond to SPC albeit in the micromolar concentration range making them unlikely as candidates for mediating the high affinity SPC effects in atrial cardiomyocytes.

As of to date, three high affinity and a number of low affinity GPCRs for SPC have been identified (Fig. 2). The first receptor responding with high affinity to SPC was GPR68, also referred to as *o*varian cancer *G* protein coupled *r*eceptor 1 (OGR1) [Xu et al., 2000]. OGR1 was reported to bind SPC with high affinity ($K_d = 33$ nM), mobilize intracellular calcium in a pertussis toxin (PTX) dependent manner, stimulate the p42/44 MAP kinase cascade as well as stereospecifically internalize the receptor. The most interesting finding, however, was that OGR1 expression conferred onto SPC the ability to inhibit cell growth in transfected HEK293 as well as various ovarian cancer cell lines.

The second high affinity SPC receptor is GPR4 sharing 51% sequence homology with OGR1. Similar to OGR1, GPR4 is a high affinity SPC receptor but it is distinct from OGR1 in that it is also activated by LPC [Zhu et al., 2001]. GPR4 activation leads to intracellular calcium mobilization, serum response element (SRE) activation, receptor internalization, extracellular signal regulated kinase (ERK) activation, and stimulation of cell migration. It is of interest to note that SPC-induced ERK activation of GPR4 is PTX-sensitive while OGR1-mediated ERK activation is insensitive to PTX treatment. Thus, despite the about 50% homology between GPR4 and OGR1, the same high affinity ligand stimulates the p42/44 MAP kinase cascade through different G protein pathways.

A receptor not activated by SPC but part of the OGR1 cluster is T cell death associated gene 8

(TDAG8). TDAG8 is specifically activated by psychosine and related glycosphingolipids such as glucosylpsychosine but not by S1P, LPA, or LPC. Psychosine has long been suspected to act through a specific G protein coupled receptor [Okajima and Kondo, 1995; Himmel et al., 1998] and has been paired with TDAG8 in 2001 [Im et al., 2001]. Psychosin attracted attention due to its implication in globoid cell leukodystrophy (GLD), a disease characterized histopathologically by apoptosis of oligodendrocytes, progressive demyelination, and the existence of large multinuclear globoid cells derived from perivascular microglia. Treatment of TDAG8-expressing HEK293 or RH7777 cells with psychosine results in formation of multinuclear, globoid cells. Although psychosine exerts its agonistic effect in the micromolar range it may still be of pathophysiological relevance in GLD given that it accumulates to micromolar levels in GLD patients.

### Low Affinity SPC Receptors

Various high affinity S1P receptors are generally also activated by SPC in the micromolar concentration range. The $S1P_1$ receptor constitutes such an example: SPC has been shown to activate $G\alpha_{i2}$ through $S1P_1$ in Sf9 cell membranes [Windh et al., 1999]. Furthermore, SPC mediates intracellular calcium mobilization via the $S1P_1$, $S1P_2$, and $S1P_3$ receptors in *Xenopus* oocytes [Ancellin and Hla, 1999]. However, in contrast to S1P, SPC is unable to bind to S1P receptors nor does it induce $S1P_1$ receptor internalization [Liu et al., 1999; Wang et al., 1999]. Currently, it is unclear which of the SPC effects are mediated via the $S1P_{1-5}$ receptor cluster or other low affinity SPC receptors as opposed to high affinity SPC receptors such as OGR1 and GPR4.

G2A, so named for its ability to cause accumulation of cells in the $G_2$/M phase of the cell cycle, is a G protein coupled receptor with dual responsiveness to LPC and SPC [Kabarowski et al., 2001]. G2A is predominantly expressed in T- and B-lymphocytes and its genetic ablation leads to the development of a late-onset autoimmunity resembling systemic lupus erythematodes [Le et al., 2001]. G2A has recently been described to promote apoptosis [Lin and Ye, 2003] and most likely disruption of G2A-mediated programmed cell death of self recognizing T-cells is responsible for the observed autoimmunity in $G2A^{-/-}$ mice. In addition, G2A was found by immunohistochemistry in macrophages within atherosclerotic plaques of human coronary artery specimens but not in fibrous plaques devoid of macrophages suggesting that G2A may be involved in the formation and/or progression of atherosclerosis [Rikitake et al., 2002]. G2A is activated by LPC and SPC in the low and medium nanomolar range, respectively [Kabarowski et al., 2001]. Both LPC and SPC activate G2A-dependent intracellular calcium mobilization, induce receptor internalization, and stimulate the ERK mitogen-activated protein (MAP) kinase pathway. However, only LPC but not SPC displays chemoattractant properties toward Jurkat T cells expressing G2A [Kabarowski et al., 2001] suggesting that SPC shares the majority but not all of the agonist properties of the high affinity ligand LPC. It is also of interest to note that G2A displays a significant level of intrinsic signaling via $G\alpha_q$ and $G\alpha_s$ pathways [Lin and Ye, 2003]. LPC does not further enhance inositolphosphate production but dose-dependently augments intracellular cAMP accumulation [Lin and Ye, 2003] as well as G2A-induced apoptosis. Thus, G2A appears to represent a receptor that displays ligand-regulated and constitutive activation in a signaling pathway specific manner. Activation by SPC of G2A was not tested in this study and it, therefore, remains to be clarified whether SPC is endowed with the ability to further enhance the various G2A signaling pathways.

### GPCRs for PA

PA has traditionally been implicated in regulating intracellular signaling events. It has, for example, been reported to be involved in membrane recruitment of Raf-1, which contains a specific PA binding region [Rizzo et al., 2000]. Besides its action as intracellular second messenger studies suggested that PA may signal via cell surface GPCRs either in the micromolar [Sliva et al., 2000] or nanomolar concentration range [Alderton et al., 2001]. Alderton et al. [2001] have shown that PA and related molecular species such as doPA activate, via a $G\alpha_{i/o}$-dependent mechanism, the p42/44 mitogen-activated protein kinase (MAPK) pathway in HEK293 cells. They suggested GPR45 to be a possible candidate but did not test PA for functional activity on GPR45 in this report. In 2003, doPA was shown to act agonistic on the closely related GPR63 receptor, albeit with low

potency [Niedernberg et al., 2003]. Notwithstanding, this was the first report for a cell surface receptor responsive to PA.

### The GPR-3, -6, and -12 Family

Human GPR3, 6, and 12 have been cloned by several independent groups as members of the GPCR family exhibiting high similarity to each other [Marchese et al., 1994; Heiber et al., 1995; Song et al., 1995]. Their closest phylogenetic neighbors are the S1P, LPA, melanocortin, and cannabinoid receptors (Fig. 2) to which they exhibit about 43, 44, and 42% similarity, respectively. Whereas initial reports suggested that GPR3,6,12 were predominantly expressed in the central nervous system it is now clear that they are abundantly expressed in the CNS and peripheral tissues [Eidne et al., 1991; Heiber et al., 1995; Uhlenbrock et al., 2003]. Early functional studies have shown that expression of human GPR3 (originally referred to as hACCA = human adenylate cyclase constitutive activator) in various mammalian cell lines conferred intrinsic generation of cAMP onto the cells in an apparently ligand-independent manner [Eggerickx et al., 1995]. Later, GPR6 and GPR12 were found to be equally capable of strong ligand-independent adelylycyclase activation thus constituting a family of highly constitutively active receptors [Uhlenbrock et al., 2002]. Recently, it was shown that S1P is capable of mobilizing intracellular $Ca^{2+}$ in GPR3, 6, and 12 expressing cells in the nanomolar range [Uhlenbrock et al., 2002]. $Ca^{2+}$ mobilization was sensitive to PTX suggesting involvement of $G\alpha_i$ type of G proteins. It has to be noted that release of $Ca^{2+}$ from intracellular stores was visible only when cells were pretreated with a certain concentration of the polyanionic compound suramin which functions as an antagonist for the $S1P_3$ and probably other S1P receptors [Ancellin and Hla, 1999]. In the same study, S1P was tested for agonism in functional cAMP assays but was unable to further enhance cAMP generation. An independent study has confirmed S1P action on GPR12 although S1P concentrations required to release $Ca^{2+}$ from intracellular stores were micromolar [Ignatov et al., 2003]. Different assay protocols and slightly different approaches to measure $Ca^{2+}$ release may be responsible for this. It is interesting to note that Ignatov et al. [2003] found GPR12 to be responsive to nanomolar concentrations of SPC, a lipid that was devoid of agonistic activity in the study from Uhlenbrock et al. [2002]. Clearly additional experiments are required to clarify the ligand nature for GPR12 and probably readdress the whole GPR3, 6, and 12 family with respect to preference for S1P or SPC.

## ANALYSIS OF UNKNOWN AND CONTENTIOUS ISSUES

### Is There Evidence for a Dual High Affinity S1P/SPC Receptor?

One of the first actions of SPC mediated via a cell surface $G\alpha_{i/o}$-linked GPCR in the nanomolar range was its potent and stereospecific activation of $I_{K.Ach}$ in guinea pig atrial myocytes, an activity shared by S1P [Buenemann et al., 1996]. Later, considerable species differences were encountered with respect to this effect [Meyer zu Heringdorf et al., 2002]. SPC hardly activated $I_{K.Ach}$ in cardiomyocytes derived from human or murine sources whereas S1P displayed nanomolar potency. Whereas the data obtained on human myocytes suggested the involvement of the $S1P_3$ receptor, it is presently unclear which receptor mediated the response to nanomolar concentrations of SPC in guinea pig. As opposed to species differences underlying the discrepant findings on S1P and SPC activity in guinea pig versus human/mouse cardiomyocytes the postulated dual high affinity S1P/SPC receptor may still be hidden among the about 170 remaining orphan receptors. Given the fact that receptors with identical ligands can display low homology on the amino acid level such as GPR63 and the $S1P_{1-5}$ family, GPR23 (P2Y9) and the $LPA_{1-3}$ family, the postulated high affinity S1P/SPC receptor linked to activation of $I_{K.Ach}$ in guinea pig cardiomyocytes may well be found out with the S1P or SPC or GPR3–6–12 cluster.

### OGR1 and GPR4: Receptors for Lipids or Protons or Both?

Very recently, OGR1, previously described as a high affinity receptor for SPC, has been reported to act as a proton sensing receptor stimulating inositolphosphate formation [Ludwig et al., 2003]. Upon transient or stable expression of OGR1 in CCL39 or HEK293 cells a robust and initially apparently ligand-independent activation of the phospholipase C-β (PLCβ) inositolphosphate signaling cascade was observed the extent of which depended on

the pH of the assay buffer. Site directed mutagenesis proved that histidines at the extracellular surface and within TMVII play a key role in pH sensing of the OGR1 receptor that is fully activated at pH 6.8 and inactive at pH 7.8. SPC, reported to be a high affinity ligand for OGR1, did neither stimulate inositolphosphate production nor did it modify the receptors ability to generate inositolphosphates when exposed to different concentrations of protons. In the same study, a very similar observation was made for GPR4. The histidine residues important for the pH—sensing ability of OGR1 are conserved in the closely related GPR4 receptor and indeed, GPR4 responded to protons as did OGR1. GPR4 and OGR1, however, differ in their pH-modulated signaling pathways: whereas OGR1 activates the Gq-PLCβ-$IP_3$ pathway, GPR4 activates the Gs-adenylylcyclase-cAMP pathway. Again, SPC, supposed to act as a high affinity ligand for GPR4 did not confer cAMP production to cells transfected with GPR4 nor did it modulate pH-dependent cAMP accumulation. Very recently, Bektas et al. [2003] reported about ligand-independent signaling of GPR4 and its inability to respond to SPC and LPC in assays monitoring arrestin translocation, GTPγS binding or internalization. These observations are intriguing as they impose the following question: what is the ligand for OGR1 and GPR4? SPC or protons or both? The fact that lipid ligands or protons, respectively activate both receptors does not exclude the existence of additional modulators of receptor function, particularly if the signaling pathways of the reported ligand classes differ. SPC-mediated calcium mobilization in GPR4 transfected cells was sensitive to PTX suggesting involvement of the $G\alpha_i$ pathway. Furthermore, GPR4-dependent activation of a SRE reporter was partly sensitive to PTX and the Rho inhibitor C3 exoenzyme and fully inhibited by a combination of both implying $G_i$ and Rho signaling reponses in SRE activation [Zhu et al., 2001]. In contrast, protons were shown to activate the Gs cAMP signaling cascade via GPR4. It may, therefore, well be that GPR4 is modulated by different ligand classes in a signal transduction specific manner. Future studies will be required to shed more light on the true nature of the ligands for both, OGR1 and GPR4.

The fact that OGR1 and GPR4 are activated by protons imposes the question whether the two related sequences of TDAG8 and G2A may be equally sensitive to modulation by protons in addition to modulation by lipid ligands. An alignment of OGR1-related receptors is shown in Figure 3. Whereas GPR4 and OGR1 are clearly endowed with a set of histidines for pH sensing, G2A only contains a single histidine in the first outer loop in a position corresponding to those of OGR1 and GPR4, unlikely to confer pH sensitivity onto the receptor. It is intriguing, though, that G2A contains positively charged amino acids (arginine, lysine) at positions equivalent to the histidines in GPR4/OGR1 (Fig. 3). Given that the GPR4/OGR1/TDAG8/G2A family displays considerable sequence similarity one might postulate that conserved residues may fulfill comparable functions in all receptors. Protons are thought to activate OGR1 and GPR4 by binding to nitrogen atoms in the histidine-imidazole ring that is otherwise engaged in a hydrogen bond interaction, thus disrupting the inactive conformation and allowing the receptor to switch from the inactive to the active state. If positive charges at the positions corresponding to the conserved hisitidines in G2A exert the same function and contribute to the receptor's active state, G2A should display considerable constitutive activity. In keeping with this hypothesis, G2A does indeed display considerable ligand independent signaling [Lin and Ye, 2003].

TDAG8 contains two histidines in the N-terminal domain and conservative histidine replacements in the first and second outer loop as well as in TMVII. It is tempting to speculate that the position of basic amino acids within TDAG8 may confer pH sensitivity to this receptor in a manner similar to OGR1 and GPR4, a hypothesis that remains to be tested.

### A Single Lipid Ligand Activates Various Structurally Unrelated Receptor Subfamilies: Has the Ability to Bind Certain Lipids Evolved More Than Once in Vertebrates?

Lipid ligands and their receptors exhibit a certain degree of promiscuity. Examples for promiscuous receptors are constituted by the $S1P_1$ receptor, OGR1, GPR4, and GPR63. The $S1P_1$ receptor is a high affinity receptor for S1P but is also activated by LPA. LPA binds to $S1P_1$ with low micromolar affinity ($K_i \sim 2$ μM), induces receptor phosphorylation, MAP kinase activation, and Rho-dependent cytoskeletal changes [Lee et al., 1998] suggesting that the $S1P_1$ receptor can serve as molecular target for

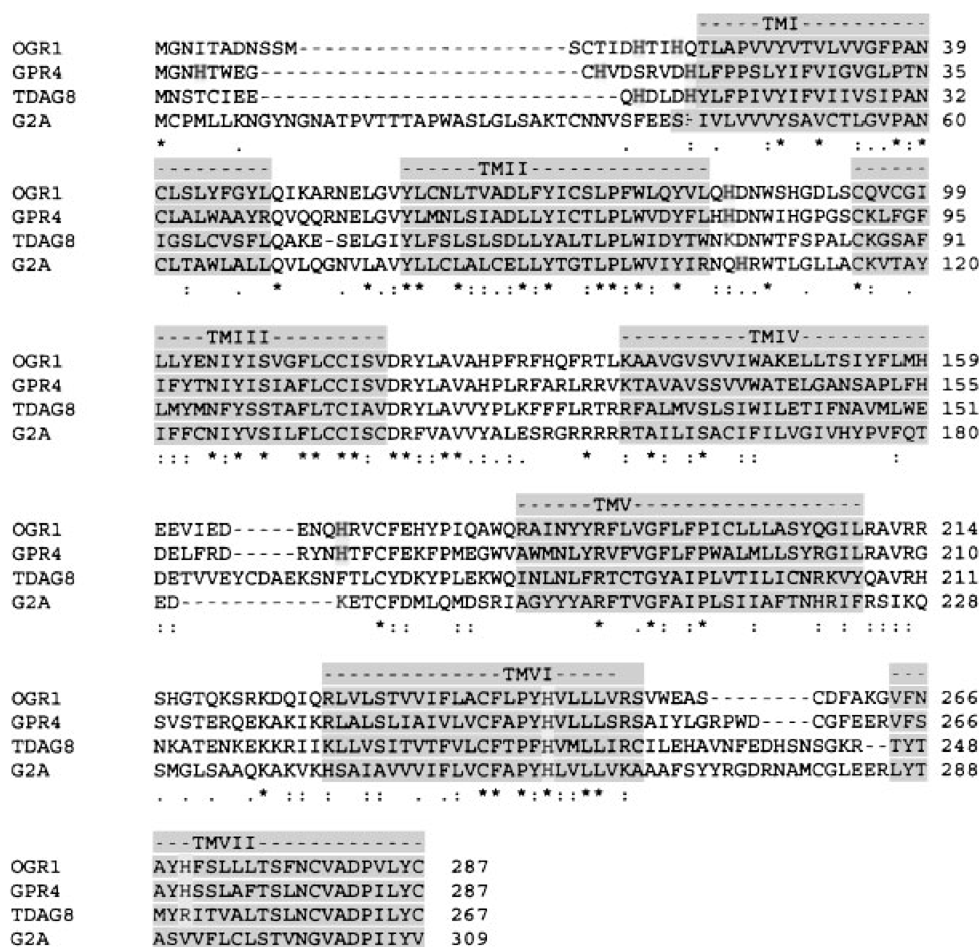


**Fig. 3.** Sequence alignment of ovarian cancer G protein coupled receptor 1 (OGR1) and the related receptors GPR4, T cell death associated gene 8 (TDAG8), and G2A. All sequences lack the C-terminal intracellular domains. The putative transmembrane regions are boxed in light blue, histidines involved in pH sensing of OGR1 and GPR4 are located in the N-terminal domain, first and second outer loops as well as in TMVII and are boxed in yellow. A histidine in TMVI conserved within the family of OGR1 and related receptors is boxed in light grey. Of interest, the psychosine receptor TDAG8 also contains histidines in the N-terminal domain in a location similar to OGR1 and GPR4. Positively charged lysines or arginines within TDAG8 or G2A in positions corresponding to histidines within GPR4/OGR1 are highlighted in red. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

LPA in addition to S1P. It should be noted that the action on the $S1P_1$ receptor of LPA is a controversially discussed issue as Windh et al. [1999] did not observe any effects of LPA in membranes of Sf9 cells coexpressing $S1P_1$ and $G\alpha_{i2}$. OGR1 was reported to be a high affinity receptor for SPC [Xu et al., 2000] in addition to being a sensor for protons [Ludwig et al., 2003]. GPR4 is a high affinity receptor for SPC and displays somewhat lower affinity for LPC with $K_d$ values of 36 and 159 nm, respectively [Zhu et al., 2001]. Like OGR4, GPR4-dependent second messenger production is sensitive to modulation by protons [Ludwig et al., 2003]. GPR63 is activated by micromolar concentrations of S1P and dhS1P but also by the structurally different doPA [Niedernberg et al., 2003]. Various S1P receptor subtypes are activated by SPC in micromolar concentrations in addition to the high affinity ligand S1P [for review see Pyne and Pyne, 2000a,b]. Conversely, a single lipid ligand can activate various structurally distinct classes of receptors as will be outlined in detail below.

Recently, two novel *Xenopus* LPA receptors named $XLPA_1$ and $XLPA_2$ have been cloned [Kimura et al., 2001]. They exhibit about 90% identity with the mammalian $LPA_2$ receptor. Overexpression of both receptors in *Xenopus* ocytes potentiated LPA-induced chloride currents, and heterologous expression of both receptors in B103 neuroblastoma cells conferred

LPA-induced cell rounding and adenylyl cyclase (AC) inhibition to the cells that otherwise are unresponsive to LPA. Together with a third high affinity *Xenopus* LPA receptor [Guo et al., 1996], these three receptors constitute a family of high affinity *Xenopus* LPA receptors. It is remarkable that XPSP24 shares only about 20% amino acid identity with the mammalian LPA receptors or $XLPA_{1/2}$. Site-directed mutagenesis studies revealed that a highly conserved glutamine residue in TMIII of $LPA_{1-3}$ is critical for interacting with the LPA hydroxyl group [Wang et al., 2001]. XPSP24 but not its mammalian orthologs GPR45 and GPR63 contain this glutamine residue (Fig. 4). Those data support the notion that XPSP24 is an LPA receptor but GPR45/63 must have lost the ability to respond to LPA during evolution. Consistent with this hypothesis, attempts to activate GPR45 and GPR63 with LPA have not been successful [Kawasawa et al., 2000b]. Very recently, the orphan receptor GPR23 (also referred to as P2Y9) has been reported to be a high affinity LPA receptor [Noguchi et al., 2003] distant from the LPA/S1P cluster. GPR23 shares highest identity with the chicken purino receptor P2Y5. GPR23 lacks the conserved glutamine in TMIII as well as a basic amino acid conserved in the $LPA_{1-3}/S1P_{1-5}$ cluster (corresponding to R277 in $LPA_3$) suggested to be involved in binding the phosphate moiety of LPA or S1P, respectively [Parrill et al., 2000; Wang et al., 2001, cf Fig. 4]. Instead, GPR23 contains a lysine at the boundary between TMVII and the third outer loop, which may indicate that LPA still uses the same basic residues like in the $LPA_{1-3}$ receptors but its binding mode is slightly distorted.

In the family of S1P receptors, a glutamate residue in TMIII supposed to ion pair with the S1P ammonium [Parrill et al., 2000] is highly conserved among all subtypes (LPA receptors contain a highly conserved glutamine at the corresponding position, cf. Fig. 4). GPR63, which is activated by S1P does not contain this conserved glutamate residue (E121) but an alanine instead (Fig. 4). Lack of the crucial glutamate in GPR63 may explain the moderate affinity to S1P. In accordance with this hypothesis, $S1P_1$ receptor mutants such as E121A are only activated by micromolar concentrations of S1P.

Taken together, the group of high affinity LPA receptors includes $LPA_{1-3}$ and the *Xenopus* homologs $XLPA_{1/2}$. However, LPA can also activate GPCRs with high affinity that are not within the $LPA_{1-3}/XLPA_{1-2}$ cluster such as xPSP24 or P2Y9/GPR23. High affinity S1P receptors include the closely related $S1P_{1-5}$ receptors but S1P also activates receptors that are outside the $S1P_{1-5}$ cluster such as GPR63 or GPR12. These data suggest that the ability of GPCRs to bind LPA or S1P and use them as transducers for extracellular signals has evolved at least twice in vertebrates.

## Constitutive Activity: A Hallmark of Certain Lipid Receptor Clusters?

It is remarkable that ligand independent signaling has been reported for the majority of receptors within and close to the S1P receptor family. Various high affinity S1P receptors display constitutive activity such as $S1P_1$, $S1P_4$, and $S1P_5$ [Lee et al., 1996; Niedernberg et al., 2003b]. The closely related GPR3, 6, and

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                -------TMIII-----------------                       ---------TMVII------------
XPSP24   107--CQISAMLYWFFVLEG-VAILLIISVDRF--133--   --305--SSFYSISTCTLW-----LTYLKSVFNPVIYCWRIKKFR--332
GPR45    107--CRLSATLYWFFVLEG-VAILLIISVDRF--133--   --305--SSFYATSTCVLW-----LSYLKSVFNPIVYCWRIKKFR--332
GPR63    154--CRVSAMFFWLFVIEG-VAILLIISIDRF--180--   --352--HNFFEISTWLLW-----LCYLKSALNPLIYYWRIKKFH--385
XLPA1    123--WLLRQGLIDTSLTAS-VANLLAIAIERH--149--   --291----ILAYEKFFLL-----LAEFNSAMNPIIYSYRDKEMS--322
XLPA2    123--WLLRQGLIDTSLTAS-VANLLAIAIERH--149--   --291----ILAYEKFFLL-----LAEFNSAMNPIIYSYRDKEMS--322
LPA1     121--WLLRQGLIDTSLTAS-VANLLAIAIERH--147--   --289----VLAYEKFFLL-----LAEFNSAMNPIIYSYRDKEMS--320
LPA2     104--WFLRQGLLDTSLTAS-VATLLAIAVERH--130--   --273----VLAVEKYFLL-----LAEANSLVNAAVYSCRDAEMR--304
LPA3     102--WFLRQGLLDSSLTAS-LTNLLVIAVERH--128--   --271----VQHVKRWFLL-----LALLNSVVNPIIYSYKDEDMY--302
GPR23    111------SGTAFLTNIYGSMLFLTCISVDRF--137--   --290--NCFLERFAKIMYPITLCLATLNCCFDPFIYYFTLESFQ--327
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**Fig. 4.** Amino acid sequence aligment of human and *Xenopus* high affinity LPA receptors as well closely related phylogenetic neighbors. Aligned are transmembrane regions III and VII and short sequence stretches of the TM cytoplasmic boundaries. Putative TM regions are shaded in light blue. Glutamine (Q) in TMIII is conserved among all high affinity LPA receptors except for GPR23 which is a high affinity LPA receptor out with the $LPA_{1-3}/XLPA_{1/2}$ cluster. XPSP24 is a putative LPA receptor and contains glutamine about one helix turn higher in TMIII. The two human XPSP24 homologs, GPR45 and GPR63 do not contain glutamine in TMIII consistent with reports showing that they are unresponsive to LPA [Kawasawa et al., 2000b]. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

12 receptors are strong intrinsic stimulators of the $G\alpha_s$-AC signaling pathway [Uhlenbrock et al., 2002]. Even the closest non-edg receptors such as the cannabinoid and the melanocortin receptors do exhibit ligand independent signaling characteristics [Bouaboula et al., 1997; Nie and Lewis, 2001; Holst and Schwartz, 2003]. Apparently, a certain branch of the phylogenetic GPCR tree is endowed with the ability to signal in an agonist-independent way. Constitutive activity does not rule out that endogeneous agonists exist and control receptor function, indeed many of the constitutively active lipid receptors possess agonists that further enhance activity at least in selected signal transduction pathways [Uhlenbrock et al., 2002; Lin and Ye, 2003]. It does, however, open up the possibility that endogeneous inverse agonists may be identified reminiscent of findings obtained in the area of virally encoded highly constitutively active GPCRs [Holst et al., 2001].

## EVIDENCE FOR ADDITIONAL LPA/S1P/PA/SPC RECEPTORS

The identification of LPA and S1P receptors structurally different from the $S1P_{1-5}$ and $LPA_{1-3}$ family implies the likelihood of identifying additional lipid receptors. This hypothesis is supported by experimental data that cannot be explained by the currently known lipid receptor repertoire as exemplified for the LPA receptor family: (i) LPA-induced responses such as inositolphosphate production, AC inhibition, stress fiber formation are strongly reduced but still detectable in embryonic fibroblasts derived from $LPA_1$/$LPA_2$ receptor double knockout mice [Contos et al., 2002]. $LPA_3$ receptor expression could not be detected in these cells and GPR23/P2Y9 expression was not tested since its association with LPA was not known at that time. (ii) RH7777 cells lacking endogeneous $LPA_{1-3}$ receptors still show mitogenic responses to LPA [Hooks et al., 2001]. (iii) The ligand preference of platelets responding to LPA analogs with aggregation is not consistent with that of $LPA_{1-3}$ or GPR23/P2Y9 [Tokumura et al., 2002; Noguchi et al., 2003]. SPC represents a signaling molecule by its own and some but not all of its effects can be explained via action through the $S1P_{1-5}$ receptor family. Future studies will show whether OGR1 and GPR4 turn out to be proton sensing receptors rather than high affinity SPC receptors and those observations may determine the need to hunt for additional SPC receptors. PA has been reported to signal through an endogeneous receptor in HEK293 cells in the nanomolar concentration range [Alderton et al., 2001]. However, only one cell surface PA receptor is known so far requiring micromolar PA concentrations for activation [Niedernberg et al., 2003a]. A high affinity PA cell surface receptor, therefore, remains to be identified.

## POTENTIAL FUTURE DIRECTIONS AND CHALLENGES

### Assigning Functions to Individual Lipid Receptors and Generating Selective Medicinal Chemistry

A spectrum of structurally closely related and distant receptors mediate the action of lysophospholipid mediators which are involved in a broad range of (patho)-physiological processes. Assigning specific functions to particular receptor subtypes has been challenging and assignments are further complicated by the fact that ligands modulate cell function from inside and outside of cells [Gobeil et al., 2003; McIntyre et al., 2003; Spiegel and Milstien, 2003]. Gene disruption studies have provided some insight into the physiological role of $S1P_1$, $S1P_2$, $LPA_1$, and G2A [Contos et al., 2000; Liu et al., 2000; MacLennan et al., 2001], but have remained inconclusive in other cases such as $LPA_2$ and $S1P_3$ [Ishii et al., 2001; Contos et al., 2002; for review see Yang et al., 2002] as knockout mice did not show any obvious phenotype. To better dissect the role of individual receptors in health and disease, selective agonists and antagonists would be required. However, medicinal chemistry has so far given rise to molecules that prefer certain receptor subtypes but do not show absolute selectivity [Heise et al., 2001; Hasegawa et al., 2003; Ohta et al., 2003]. Given the overlapping expression and redundant signaling pathways of many lipid receptors of the S1P family [Van Brocklyn et al., 2002] together with the observation that gene ablation of certain receptor subtypes is tolerated without obvious phenotypic abnormalities [Yang et al., 2002], it is tempting to speculate that receptors are partly redundant and highly selective medicinal chemistry would not be a prerequisite for a successful therapeutic agent.

### Lipid Receptor Homo- and Hetero-Dimerization: Adding an Additional Level of Complexity to Analysis of Receptor Function

In the last years, analysis of GPCR dimerization has become a popular and productive area of research and considerable evidence is available to suggest that GPCRs can form and function as homo- or hetero-dimers [Bouvier, 2001; Gomes et al., 2001; Milligan, 2001; Angers et al., 2002]. Various studies on the function of S1P receptors suggest that biological responses to S1P in cells depend on the expression of various rather than a single S1P receptor [for review see van Brocklyn et al., 2002] and it appears that S1P receptors interact to mediate their responses in a collaborative effort. It is unclear, however, at which level of the signaling cascade interaction takes place, i.e., whether receptors themselves interact on the level of the plasma membrane or whether they activate signaling pathways in an overlapping fashion. At least for the high affinity $S1P_{1-5}$ receptor family $S1P_1$ is reported to form homodimers as well as hetero-dimers with $S1P_3$ and $S1P_2$ [Van Brocklyn et al., 2002]. Likewise, $S1P_2$ was shown to dimerize with $S1P_3$, in all cases S1P did not have an effect on dimer formation. Interestingly, $S1P_1$ does not form dimers with $LPA_1$ as judged by immunoprecipitation studies and $LPA_1$ does not seem to dimerize with $S1P_1$ or $S1P_3$. Thus, no data are available yet supporting the assumption that different clusters of lipid receptors dimerize with each other, nonetheless, it is an intriguing possibility that still requires experimental verification.

To which extent dimerization obscures the analysis of S1P/lipid receptor function in general in transfected cells can currently not be answered satisfactorily. Given the abundant and overlapping expression of many receptors within a single ligand family, we may observe dimer signaling although individual receptor cDNAs are used for transfection. One might argue that overexpression of the examined receptor is well beyond endogeneous expression and thus should not impose major concerns as to whether we observe monomer- or dimer-signaling. Nevertheless, the potential effects of hetero-dimer formation should be kept in mind when interpreting data generated with lipid receptors in cell lines endogeneously endowed with a spectrum of potential dimer partners. Unfortunately, development of pharmacological tools such as selective receptor agonists and antagonists is still in its infancy but may represent a promising strategy to study ligand binding, signaling and trafficking of homo- and hetero-meric lysophospholipid receptor complexes in the future.

## CONCLUDING REMARKS

Identification of the S1P1 (edg1) receptor as a high affinity S1P receptor has triggered an immense effort in trying to elucidate how lysophospholipid mediators interact with cells, not only from an intracellular but particularly from an extracellular perspective. Whereas the role of the $S1P_1$ receptor and its implication in angiogenesis/vascular maturation/cancer is becoming increasingly clear, much less is known about the other high and low affinity S1P or closely related lipid receptors. The pleiotropic nature of many lysophospholipids combined with the widespread and overlapping expression of their receptors imposes a serious challenge for biological validation. Undoubtedly, identification of the complete set of lipid receptors as well as development of specific lipid receptor agonists and antagonists will aid to more accurately delineate the roles of these receptor in signaling processes in health and disease.

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