Mol. Cells *29*, 99-104, February 28, 2010
DOI/10.1007/s10059-010-0041-z

Minireview

Molecules and Cells

Sphingosine-1-Phosphate Receptors: Zooming in on Ligand-Induced Intracellular Trafficking and Its Functional Implications

Dennis Verzijl, Stephan L.M. Peters, and Astrid E. Alewijnse*

Regulatory processes including receptor phosphorylation and intracellular trafficking, also referred to as receptor internalization, are important processes to terminate G protein-coupled receptor (GPCR) signaling. Compelling evidence now indicates that internalization of a receptor is not necessarily the endpoint of signaling, but can also be the beginning of the activation of intracellular signaling pathways.

Sphingosine-1-phosphate (S1P) receptors, which are activated by the endogenous phospholipid S1P, belong to the family of GPCRs. Interestingly, there is evidence indicating differential intracellular trafficking of one of the S1P receptor subtypes, the $S1P_1$ receptor, upon agonist activation by either S1P or the synthetic agonist FTY720-P. Moreover, the differential effect of FTY720-P on $S1P_1$ receptor regulation has been suggested to be the mechanism of action of this drug, which is now in Phase III clinical trials for the treatment of multiple sclerosis. It is thus of importance to get a good insight into the regulation of S1P receptors. This review therefore gives a detailed overview about the current state of knowledge on S1P receptor internalization and its functional implications, including some data on nuclear signaling of S1P receptors.

INTRODUCTION

The bioactive sphingolipid S1P regulates a wide variety of physiological processes in the cardiovascular (Peters and Alewijnse, 2007; Takuwa et al., 2008) and immune system (Rivera et al., 2008). The cellular targets of S1P were discovered approximately 10 years ago and revealed to be five G protein-coupled receptors (GPCRs), named $S1P_1$ to $S1P_5$ (Chun et al., 2002). GPCRs constitute a large family of transmembrane receptors that convert extracellular stimuli ranging from photons, ions, amines, peptides and phospholipids into intracellular responses (Luttrell, 2006; Rosenbaum et al., 2009). The $S1P_1$, $S1P_2$ and $S1P_3$ receptor, formerly referred to as endothelial differentiation genes EDG-1, EDG-5 and EDG-3, are widely expressed and are abundant in the cardiovascular system (Alewijnse and Peters, 2008; Takuwa et al., 2008). The $S1P_1$ receptor plays a critical role in angiogenesis and vascular maturation, and $S1P_1$ knockout mice die prenatally due to vascular leakage as a consequence of incomplete vascular maturation (Liu et al., 2000). Knockout models of $S1P_2$, $S1P_3$ and $S1P_5$ do not reveal such severe phenotypes (Choi et al., 2008) although the $S1P_2$ receptor was found to be essential for auditory and vestibular function (MacLennan et al. 2006). The $S1P_4$ and $S1P_5$ receptor show a more specific expression pattern. The $S1P_4$ receptor is mainly expressed in the hematopoietic system and $S1P_5$ can be found in white matter tracts in the brain.

Research on S1P receptors is explosively growing since the last years, mainly due to the fact that a promising new immunosuppressive drug, which targets this class of receptors, is in Phase III clinical studies for the treatment of multiple sclerosis (Brinkmann, 2009; Mansoor and Melendez, 2008). This drug, FTY720 or Fingolimod, is after its phosphorylation to FTY720-P an agonist for all S1P receptors except the $S1P_2$ receptor (Brinkmann et al., 2002). The immunosuppressive effects of FTY720 including lymphopenia seem to be mainly $S1P_1$ receptor-mediated (Sanna et al., 2004). This receptor seems to play an important role in lymphocyte trafficking and its presence on T lymphocytes is crucial for the exit of these immune cells from the lymph nodes into the circulation (Matloubian et al., 2004). Interestingly, there are indications that FTY720-P regulates receptor expression by directing internalized $S1P_1$ receptors to the degradative lysosomal pathway (Jo et al., 2005; Oo et al., 2007). In contrast, $S1P_1$ receptors stimulated with the endogenous ligand S1P are recycled back to the plasma membrane upon internalization. Consequently, a FTY720-P-induced loss of $S1P_1$ expression on lymphocytes was suggested to be the mechanism of the observed lymphopenia. However, there are also reports suggesting that $S1P_1$ receptor agonism is sufficient to induce immunosuppressive effects (Rosen et al., 2009). Because changes in S1P receptor regulation appear to be of major importance, this review zooms in on regulation and intracellular trafficking of S1P receptors, and highlights the most important findings in this rapidly evolving field.

Regulation of S1P receptors

GPCRs, including S1P receptors, are subject to various regulatory processes, which may result in their removal from the cell

Department Pharmacology and Pharmacotherapy, Academic Medical Center, Amsterdam, the Netherlands
*Correspondence: A.E.Alewijnse@amc.uva.nl

Received December 24, 2009; accepted December 27, 2009; published online January 29, 2010

Keywords: FTY720, internalization, nuclear signaling, phosphorylation, SEW2871, S1P receptors

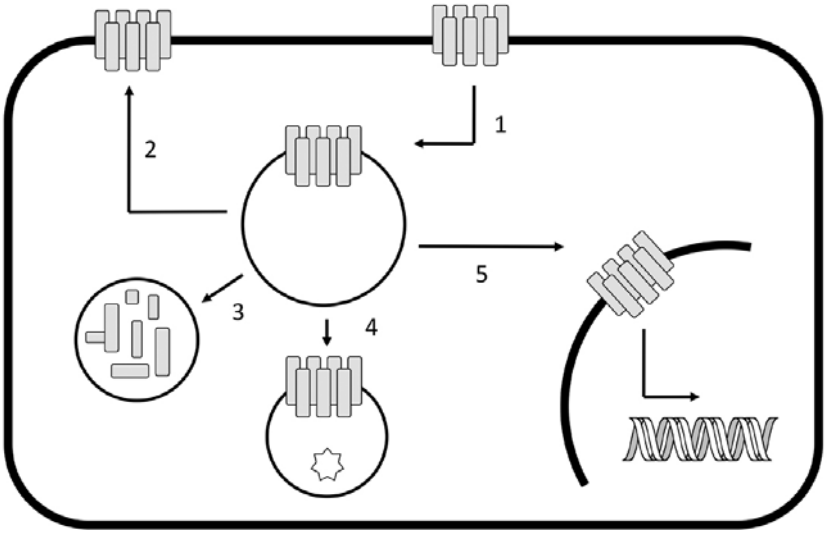

Fig. 1. Regulation of GPCRs. Upon binding of a ligand the receptor is internalized to intracellular compartments (1) and recycled to the plasma-membrane (2) or degraded (3). New insights suggest that signaling might continue in intracellular compartments (4) or in the nucleus (5).

membrane by internalization (see for extensive review Ferguson, 2001; Hanyaloglu and von Zastrow, 2008; Hendriks-Balk et al., 2008). One of the first steps in receptor regulation is receptor phosphorylation by specific GPCR kinases (GRKs), which occurs within seconds to minutes after activation (Reiter and Lefkowitz, 2006). In addition, activation leads to the recruitment and binding of beta-arrestins to the agonist-occupied receptors (Luttrell and Lefkowitz, 2002). Together these processes are referred to as desensitization and lead to the uncoupling of the receptors from G proteins and promote their internalization (Ferguson, 2001). GPCRs are also subject to heterologous phosphorylation by second messenger-activated kinases, a process which can even occur in the absence of agonist occupation and also results in receptor internalization (Chuang et al., 1996).

Internalized receptors are either recycled back to the plasma membrane (resensitization) or transported to lysosomes for degradation (down-regulation) (Fig. 1). Although receptor internalization was traditionally regarded as a way to terminate cellular signaling (Luttrell and Lefkowitz, 2002), more and more studies indicate that internalization may also be the first step in the activation of intracellular signaling pathways, including for example the MAPK signaling pathway (Daaka et al., 1998; Hanyaloglu and von Zastrow, 2008). In addition, GPCRs can translocate to the nucleus upon internalization and activate nuclear signaling pathways, a new and intriguing phenomenon in the GPCR-field (Boivin et al., 2008; Gobeil et al., 2006).

The regulatory processes of S1P receptors are best studied for the $S1P_1$ receptor and information on regulation of the other receptor subtypes is rather limiting. Upon activation, the $S1P_1$ receptor is phosphorylated on serine and threonine residues by GRKs (Watterson et al., 2002) as well as by the second messenger-activated kinase Akt/PKB (Lee et al., 2001). *In vitro* experiments suggest a GRK2 but not GRK5 mediated phosphorylation of the C-terminal tail of the receptor, which occurs within seconds after activation (Watterson et al., 2002). In contrast, phosphorylation by Akt/PKB seems to specifically occur at a threonine residue in the third intracellular loop (Thr236) of the $S1P_1$ receptor (Lee et al., 2001) (Fig. 2). Phosphorylation by Akt/PKB is important to direct receptor signaling to Rac activation, cortical actin assembly, and chemotaxis. The $S1P_1$ receptor is also subject to heterologous phosphorylation by protein kinase C (PKC) (Watterson et al., 2002). PKC-mediated phosphorylation of the $S1P_1$ receptor also involves the C-terminal tail but is much slower than the phosphorylation induced by GRKs, and occurs within minutes after activation (Watterson et al., 2002). As generally observed for GPCRs, phosphorylation of the $S1P_1$ receptor leads to uncoupling and a subsequent desensitization of $S1P_1$ receptor signaling (Xin et al., 2004).

For various GPCRs phosphorylation precedes and is a prerequisite for receptor internalization. For the $S1P_1$ receptor S1P-induced internalization is half-maximal at 15 min (Liu et al., 1999) and requires ligand concentrations that are significantly higher than those required to induce half-maximal effects on signal transduction responses (Watterson et al., 2002). These results thus suggest that $S1P_1$ receptor internalization is preceded by phosphorylation, which is half-maximal at 1 min (Watterson et al., 2002). However, a mutant $S1P_1$ receptor without the last 12 C-terminal amino acids (Fig. 2) can still be phosphorylated, but does not show any S1P-induced internalization. Based on these results it can be concluded that phosphorylation and internalization require partly overlapping but divergent pathways (Watterson et al., 2002).

Besides internalization of the $S1P_1$ receptor by the endogenous ligand S1P, also activation by synthetic ligands leads to receptor internalization. The phosphorylated analogue of FTY720 (FTY720-P) induces a concentration- and time-dependent internalization and down-regulation of the $S1P_1$ receptor (Jo et al., 2005; Müllershausen et al., 2009; Oo et al., 2007). The effects of the $S1P_1$-selective agonist SEW2871 on $S1P_1$ receptor internalization are less well investigated. Compared to S1P the affinity and potency of SEW2871 at the $S1P_1$ receptor are significantly lower (Jo et al., 2005). In line with this the concentrations of SEW2871 needed to induce internalization are higher than those of S1P (Jo et al., 2005; Jongsma et al., 2007a; Oo et al., 2007).

Research on regulation of the $S1P_2$ receptor is limited to some data on $S1P_2$ receptor internalization. As found for the $S1P_1$ receptor, the $S1P_2$ receptor is also internalized upon exposure to S1P (Jongsma et al., 2007b). In addition, activation of mast cells, which leads to the activation of sphingosine kinase 1 and subsequently to intracellular S1P generation, was shown to induce $S1P_2$ receptor internalization (Jolly et al., 2004). The intracellular produced S1P is most likely transported to the outside of the cell by ATP binding cassette (ABC) transporters (Mitra et al., 2006). As for now there is no information on the precise receptor domains and mechanisms involved in $S1P_2$

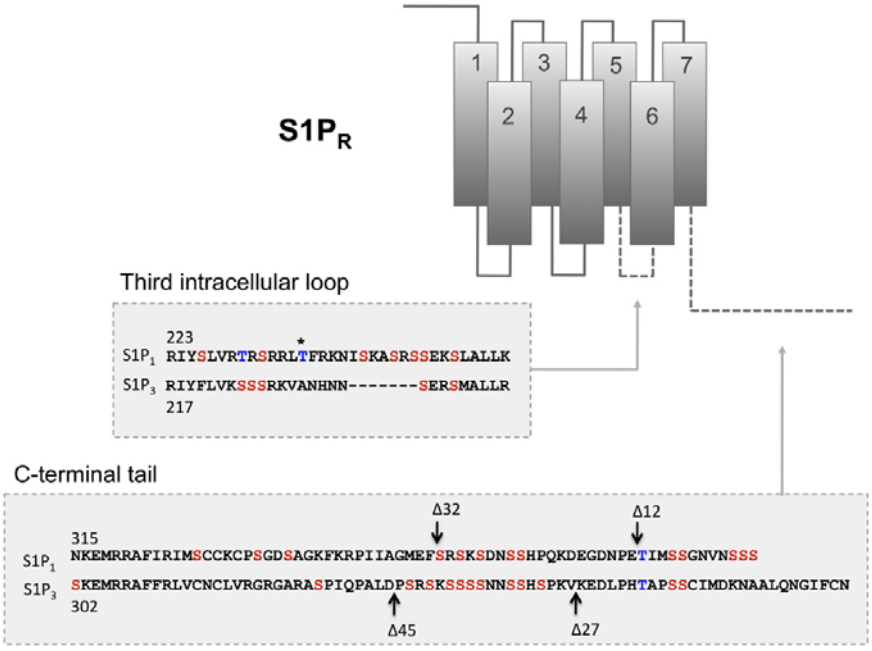

Fig. 2. Alignment of the third intracellular loop and the C-terminal tail of the human $S1P_1$ and $S1P_3$ receptor. The possible phosphorylation sites are indicated in red (serine) and blue (threonine). The arrow indicates the position at which deletion mutants have been created for both of these receptors. The * indicates the Akt/PKB phosphorylation site.

receptor internalization. Besides the effect of S1P on internalization, there is also one study describing the effect of unphosphorylated FTY720 on $S1P_2$ receptor regulation (Gräler et al., 2004). However, FTY720, either in the phosphorylated or unphosphorylated form, is devoid of any affinity for the $S1P_2$ receptor (Brinkmann et al., 2002), and the mechanism behind the observed effects remains unclear. In addition, using a different technique, we have never observed $S1P_2$ receptor internalization by FTY720 or FTY720-P in our laboratory whereas within the same set of experiments S1P induced a concentration-dependent $S1P_2$ receptor internalization (unpublished observations).

Like for the $S1P_2$ receptor, information on regulation of the $S1P_3$ receptor is rather limiting. However, there are indications that the regulation of this receptor is quite different from that of the $S1P_1$ receptor (Jongsma et al., 2007b; Rutherford et al., 2005). In contrast to the $S1P_1$ receptor, the $S1P_3$ receptor is not subject to heterologous phosphorylation, but is only phosphorylated upon activation by an agonist (Rutherford et al., 2005). The phosphorylation takes place in a stretch of amino acids located between the last 45 and 27 amino acid residues of the C-terminal tail (Fig. 2). Interestingly, the data by Rutherford et al. (2005) suggest that the last 27 amino acid residues of this tail exhibit an inhibitory effect on receptor phosphorylation. At this moment the kinase that is involved in the phosphorylation of the $S1P_3$ receptor has not been identified yet.

Regarding internalization, the $S1P_3$ receptor also displays some quite unique features. In contrast to the $S1P_1$ and many other GPCRs, internalization of the $S1P_3$ receptor seems to be independent of phosphorylation (Rutherford et al., 2005). In addition, $S1P_3$ receptor internalization is reported to be much faster than $S1P_1$ receptor internalization (Jongsma et al., 2007b). Besides S1P, also FTY720-P and several FTY720-P derivatives are reported to induce a concentration-dependent $S1P_3$-receptor internalization (Jongsma et al., 2009).

The $S1P_4$ and $S1P_5$ receptor are, compared to the other S1P receptor subtypes, less well studied and characterized. Information on $S1P_4$ receptor regulation specifically is absent whereas there is only one study on $S1P_5$ receptor regulation (Niedernberg et al., 2003). This study shows that the $S1P_5$ receptor is constitutively active and that it is mainly located in intracellular compartments, probably due to constitutive internalization. The small percentage of the receptor located in the plasma membrane is however still subject to regulatory process and is internalized upon exposure to S1P (Niedernberg et al., 2003). In addition, there is some preliminary evidence that FTY720 can cause $S1P_5$ receptor regulation (Gräler et al., 2004).

Internalization and S1P receptor recycling or degradation

Upon removal of the endogenous ligand S1P, internalized $S1P_1$ receptors recycle back to the cell membrane within hours (Jo et al., 2005; Liu et al., 1999; Müllershausen et al., 2009; Oo et al., 2007; Tao et al., 2009). In contrast, as described in the introduction, FTY720-P-induced $S1P_1$ receptor internalization does not lead to receptor recycling upon ligand removal, but seems to result in irreversible receptor degradation (Jo et al., 2005; Oo et al., 2007) and thus a decrease in $S1P_1$ membrane expression (Krump-Konvalinkova et al., 2008). S1P and FTY720-P, two compounds that both behave as full agonists at the $S1P_1$ receptor, thus differentially affect receptor fate after internalization. This difference in receptor fate is most likely a direct reflection of a different receptor ubiquitinylation upon activation by these ligands. Activation of the $S1P_1$ receptor by FTY720-P is reported to induce poly-ubiquitinylation whereas activation by S1P generally results in mono-ubiquitinylation with a small fraction of the receptors showing poly-ubiquitinylation (Oo et al., 2007; Rosen et al., 2009). At the moment it is not clear what causes this difference in ubiquitinylation status. Probably, S1P and FTY720-P stabilize different conformations of the $S1P_1$ receptor, a phenomenon also observed at other GPCRs and referred to as conformational selectivity, functional selectivity or ligand-directed signaling (Kenakin, 2007; Michel and Alewijnse, 2007). In addition, it cannot be excluded that the differences in regulation are the result of differences in ligand stability, as S1P is rapidly broken down when added to cells (Le Stunff et al., 2002). Interestingly, as described in the introduction the differential $S1P_1$ receptor regulation observed for FTY720-P has been suggested to be the mechanism of action of the novel immunosuppressive drug Fingolimod (FTY720). The phos-

phorylated form of this drug, FTY720-P, induces lymphopenia by blocking lymphocyte egress from lymph nodes. Because lymphopenia was also observed after knockout of the $S1P_1$ receptor from lymphocytes (Matloubian et al., 2004), FTY720-P-induced $S1P_1$ receptor degradation, also referred to as "functional antagonism", was thought to underlie the immunosuppressive properties of FTY720. However, there are also data supporting the hypothesis that degradation of $S1P_1$ is not necessarily involved, but that activation of $S1P_1$ alone is the most important nominator for the immunomodulatory effects observed for S1P agonists (Müllerhausen et al., 2008; Rosen et al., 2009). S1P and SEW2871, agonists that do not induce receptor degradation, are also reported to cause lymphopenia. Furthermore, $S1P_1$ antagonists do not induce immunomodulatory effects themselves, but inhibit the agonist-induced effects on immune function (Rosen et al., 2009). To this end it is important to add that the effects of SEW2871 on receptor fate are not extensively studied yet and this would certainly help the debate on "functional antagonism" versus agonism to explain the immunesuppressive effects.

Although the receptor fate upon internalization has been extensively investigated for the $S1P_1$ receptor, such studies are completely lacking for other S1P receptor subtypes. This is somewhat remarkable as FTY720-P also targets the $S1P_{3\text{-}5}$ receptors. The $S1P_3$ receptor, for example, is suggested to induce protective effects in blood vessels (Alewijnse and Peters, 2008). Sustained internalization and subsequent degradation of the $S1P_3$ receptor can thus be speculated to induce unwanted effects in the vascular system. However, first experiments in our laboratory indicate that, in contrast to the $S1P_1$ receptor, the $S1P_3$ receptor is not degraded upon activation by FTY720-P (unpublished observations).

Internalization and intracellular S1P receptor signaling

As described for other GPCRs, internalized S1P receptors can also be involved in activating intracellular signaling pathways. Compelling evidence for signaling of intracellular S1P receptors exists for the $S1P_1$ and $S1P_5$ receptor (Estrada et al., 2008; Gillies et al., 2009; Müllershausen et al., 2009). For the other S1P receptor subtypes studies on this topic are unfortunately lacking.

Recently, an extensive study on $S1P_1$ receptor activation, desensitization and trafficking revealed evidence for signaling of internalized $S1P_1$ receptors (Müllershausen et al., 2009). Interestingly, this signaling by internalized $S1P_1$ receptors, also referred to as persistent signaling, was only observed following a transient exposure to FTY720-P and not to S1P. In addition, the persistent signaling was only observed at the level of adenylyl cyclase inhibition and ERK phosphorylation and not for calcium signaling, suggesting that this last signaling pathway depends on plasma membrane receptors and not intracellular receptors (Müllershausen et al., 2009). There is also convincing evidence suggesting a role for internalized $S1P_1$ receptors in the regulation of the transcription of some growth factors. By using a wide variety of techniques including confocal and electron microscopy, Estrada and coworkers showed that endothelial $S1P_1$ receptors are rapidly internalized upon activation by S1P and translocated to nuclear regions (Estrada et al., 2008). These nuclearized $S1P_1$ receptors subsequently induce the expression of two growth factors involved in angiogenic responses. In line with these findings nuclear translocation of endogenously expressed plasma membrane $S1P_1$ receptors was also observed upon T cell activation (Liao et al., 2007). Interestingly, this relocalization of the $S1P_1$ receptor was reported to be involved in re-directing migratory responses of T-cells to non-migratory functions such as proliferation.

In addition to $S1P_1$ receptors it was recently shown that also $S1P_5$ receptors can be localized in intracellular compartments adjacent to the nucleus, namely in centrosomes (Gillies et al., 2009). $S1P_5$ receptors in centrosomes may be involved in the regulation of cell division (Gillies et al., 2009). Sphingosine kinase 1 and 2 (SK1 and SK2), which synthesize S1P, were found to co-localize with $S1P_5$ in the centrosome, suggesting *ad hoc* synthesis of the ligand. Interestingly, $S1P_5$, SK1 and SK2 are all highly expressed in the brain, suggesting an important role for intracellular $S1P_5$ receptors. Also for the $S1P_5$ receptor the subcellular localization is subject to agonist-induced regulation. However, for these receptors S1P stimulation resulted in a decreased expression of $S1P_5$ in the centrosome and increased expression of $S1P_5$ in the plasma membrane. As described above, there are no data yet on the intracellular signaling of the other S1P receptor subtypes.

CONCLUSION

As observed for other GPCRs, ligand-induced intracellular trafficking is also important for S1P receptors. Interestingly, as clearly indicated for the $S1P_1$ receptor, the functional implications of receptor internalization can differ between certain ligands and are thus ligand-dependent (Kelly et al., 2008). As becomes evident from this review studies on S1P receptor regulation mainly focus on the $S1P_1$ receptor, most likely because this receptor is suggested to be the most important target for the novel immunosuppressive drug FTY720. This review also describes some first but convincing data on nuclear signaling of S1P receptors. This intriguing aspect of S1P signaling will certainly get a lot of attention in the next years as these nuclear sphingolipid-receptors may present the proposed (Meyer zu Heringdorf, 2004) but yet unidentified intracellular targets for S1P.

In conclusion, this review clearly shows that our understanding of S1P receptor trafficking is rapidly increasing. However, many of the studies have been performed using recombinant expression systems and not primary cells. To get a good insight into intracellular trafficking of S1P receptors and its functional consequences studies on endogenous receptors are essential and this will therefore be the most important challenge for the future.

REFERENCES

Alewijnse, A.E., and Peters, S.L.M. (2008). Sphingolipid signaling in the cardiovascular system: good, bad or both? Eur. J. Pharmacol. *585*, 292-302.

Boivin, B., Vaniotis, G., Allen, B.G., and Hébert, T.E. (2008). G protein-coupled receptors in and on the cell nucleus: a new signaling paradigm? J. Recept. Signal Transduct. Res. *28*, 15-28.

Brinkmann, V. (2009). FTY720 (fingolimod) in Multiple Sclerosis: therapeutic effects in the immune and the central nervous system. Br. J. Pharmacol. *158*, 1173-82.

Brinkmann, V., Davis, M.D., Heise, C.E., Albert, R., Cottens, S., Hof, R., Bruns, C., Prieschl, E., Baumruker, T., Hiestand, P., et al. (2002). The immune modulator FTY720 targets sphingosine 1-phosphate receptors. J. Biol. Chem. *277*, 21453-21457.

Choi, J.W., Lee, C.W., and Chun, J. (2008). Biological roles of lysophospholipid receptors revealed by genetic null mice: an update. Biochim. Biophys. Acta *1781*, 531-539.

Chuang, T.T., Iacovelli, L., Sallese, M., and De Blasi, A. (1996). G protein-coupled receptors: heterologous regulation of homologous desensitization and its implications. Trends Pharmacol. Sci. *17*, 416-421.

Chun, J., Goetzl, E.J., Hla, T., Igarashi, Y., Lynch, K.R., Moolenaar, W., Pyne, S., and Tigyi, G. (2002). International Union of Pharmacology. XXXIV. Lysophospholipid receptor nomenclature. Pharmacol. Rev. *54*, 265-269.

Daaka, Y., Luttrell, L.M., Ahn, S., Della Rocca, G.J., Ferguson, S.S., Caron, M.G., and Lefkowitz, R.J. (1998) Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. J. Biol. Chem. *273*, 685-688.

Estrada, R., Wang, L., Jala, V.R., Lee, J.F., Lin, C.Y., Gray, R.D., Haribabu, B., and Lee, M.J. (2009). Ligand-induced nuclear translocation of S1P(1) receptors mediates Cyr61 and CTGF transcription in endothelial cells. Histochem. Cell. Biol. *131*, 239-249.

Ferguson, S.S. (2001). Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. Pharmacol. Rev. *53*, 1-24.

Gillies, L., Lee, S.C., Long, J.S., Ktistakis, N., Pyne, N.J., and Pyne, S. (2009). The sphingosine 1-phosphate receptor 5 and sphingosine kinases 1 and 2 are localised in centrosomes: possible role in regulating cell division. Cell Signal. *21*, 675-684.

Gobeil, F., Fortier, A., Zhu, T., Bossolasco, M., Leduc, M., Grandbois, M., Heveker, N., Bkaily, G., Chemtob, S., and Barbaz, D. (2006). G-protein-coupled receptors signaling at the cell nucleus: an emerging paradigm. Can. J. Physiol. Pharmacol. *84*, 287-297.

Gräler, M.H., and Goetzl, E.J. (2004). The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G-protein-coupled receptors. FASEB J. *18*, 551-553.

Hanyaloglu, A.C., and von Zastrow, M. (2008). Regulation of GPCRs by endocytic membrane trafficking and its potential implications. Annu. Rev. Pharmacol. Toxicol. *48*, 537-568.

Hendriks-Balk, M.C., Peters, S.L., Michel, M.C., and Alewijnse, A.E. (2008). Regulation of G protein-coupled receptor signaling: focus on the cardiovascular system and regulator of G protein signaling proteins. Eur. J. Pharmacol. *585*, 278-291.

Jo, E., Sanna, M.G., Gonzalez-Cabrera, P.J., Thangada, S., Tigyi, G., Osborne, D.A., Hla, T., Parrill, A.L., and Rosen, H. (2005). $S1P_1$-selective in vivo-active agonists from high-throughput screening: off-the-shelf chemical probes of receptor interactions, signaling, and fate. Chem. Biol. *12*, 703-715.

Jolly, P.S., Bektas, M., Olivera, A., Gonzalez-Espinosa, C., Proia, R.L., Rivera, J., Milstien, S., and Spiegel, S. (2004). Transactivation of sphingosine-1-phosphate receptors by FcepsilonRI triggering is required for normal mast cell degranulation and chemotaxis. J. Exp. Med. *199*, 959-970.

Jongsma, M., Florczyk, U.M., Hendriks-Balk, M.C., Michel, M.C., Peters, S.L., and Alewijnse, A.E. (2007a). Validation of a rapid, non-radioactive method to quantify internalisation of G-protein coupled receptors. Naunyn Schmiedebergs Arch. Pharmacol. *375*, 329-336.

Jongsma, M., Florczyk, U.M., Hendriks-Balk, M.C., van Loenen, P.B., Michel, M.C., Peters, S.L.M., and Alewijnse, A.E. (2007b). Internalisation of sphingosine-1-phosphate receptors. Naunyn. Schmiedebergs Arch. Pharmacol. *374*, 332-332.

Jongsma, M., van Unen, J., van Loenen, P.B., Michel, M.C., Peters, S.L., and Alewijnse, A.E. (2009). Different response patterns of several ligands at the sphingosine-1-phosphate receptor subtype 3 ($S1P_3$). Br. J. Pharmacol. *156*, 1305-1311.

Kelly, E., Bailey, C.P., and Henderson, G. (2008). Agonist-selective mechanisms of GPCR desensitization. Br. J. Pharmacol. *153*, S379-388.

Kenakin, T. (2007). Functional selectivity through protean and biased agonism: who steers the ship? Mol. Pharmacol. *72*, 1393-1401.

Krump-Konvalinkova, V., Chwalla, I., and Siess, W. (2008). FTY720 inhibits S1P-mediated endothelial healing: relationship to S1P1-receptor surface expression. Biochem. Biophys. Res. Commun. *370*, 603-608.

Le Stunff, H., Peterson, C., Liu,. H., Milstien, S., and Spiegel, S. (2002). Sphingosine-1-phosphate and lipid phosphohydrolases. Biochim. Biophys. Acta *1582*, 8-17.

Lee, M.J., Thangada, S., Paik, J.H., Sapkota, G.P., Ancellin, N., Chae, S.S., Wu, M., Morales-Ruiz, M., Sessa, W.C., Alessi, D.R., et al. (2001). Akt-mediated phosphorylation of the G protein-coupled receptor EDG-1 is required for endothelial cell chemotaxis. Mol. Cell *8*, 693-704.

Liao, J.J., Huang, M.C., Gräler, M., Huang, Y., Qiu, H., and Goetzl, E.J. (2007). Distinctive T cell-suppressive signals from nuclearized type 1 sphingosine 1-phosphate G protein-coupled receptors. J. Biol. Chem. *282*, 1964-1972.

Liu, C.H., Thangada, S., Lee, M.J., Van Brocklyn, J.R., Spiegel, S., and Hla, T. (1999). Ligand-induced trafficking of the sphingosine-1-phosphate receptor EDG-1. Mol. Biol. Cell *10*, 1179-1190.

Liu, Y., Wada, R., Yamashita, T., Mi, Y., Deng, C.X., Hobson, J.P., Rosenfeldt, H.M., Nava, V.E., Chae, S.S., Lee, M.J., et al. (2000). Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. J. Clin. Invest. *106*, 951-961.

Luttrell, L.M. (2006). Transmembrane signaling by G protein-coupled receptors. Methods Mol. Biol. *332*, 3-49.

Luttrell, L.M., and Lefkowitz, R.J. (2002). The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. J. Cell Sci. *115*, 455-465.

MacLennan, A.J., Benner, S.J., Andringa, A., Chaves, A.H., Rosing, J.L., Vesey, R., Karpman, A.M., Cronier, S.A., Lee, N., Erway, L.C., et al. (2006). The $S1P_2$ sphingosine 1-phosphate receptor is essential for auditory and vestibular function. Hear. Res. *220*, 38-48.

Mansoor, M., and Melendez, A.J. (2008). Recent trials for FTY720 (fingolimod): a new generation of immunomodulators structurally similar to sphingosine. Rev. Recent Clin. Trials *3*, 62-69.

Matloubian, M., Lo, C.G., Cinamon, G., Lesneski, M.J., Xu, Y., Brinkmann, V., Allende, M.L., Proia, R.L., and Cyster, J.G. (2004). Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature *427*, 355-360.

Meyer zu Heringdorf, D. (2004). Lysophospholipid receptor-dependent and -independent calcium signaling. J. Cell. Biochem. *92*, 937-948.

Michel, M.C., and Alewijnse, A.E. (2007). Ligand-directed signaling: 50 ways to find a lover. Mol. Pharmacol. *72*, 1097-1099.

Mitra, P., Oskeritzian, C.A., Payne, S.G., Beaven, M.A., Milstien, S., and Spiegel, S. (2006). Role of ABCC1 in export of sphingosine-1-phosphate from mast cells. Proc. Natl. Acad. Sci. USA *103*, 16394-16399.

Müllershausen, F., Zecri, F., Cetin, C., Billich, A., Guerini, D., and Seuwen, K. (2009). Persistent signaling induced by FTY720-phosphate is mediated by internalized $S1P_1$ receptors. Nat. Chem. Biol. *5*, 428-434.

Niedernberg, A., Blaukat, A., Schöneberg, T., and Kostenis, E. (2003). Regulated and constitutive activation of specific signaling pathways by the human $S1P_5$ receptor. Br. J. Pharmacol. *138*, 481-493.

Oo, M.L., Thangada, S., Wu, M.T., Liu, C.H., Macdonald, T.L., Lynch, K.R., Lin, C.Y., and Hla T. (2007). Immunosuppressive and anti-angiogenic sphingosine 1-phosphate receptor-1 agonists induce ubiquitinylation and proteasomal degradation of the receptor. J. Biol. Chem. *282*, 9082-9089.

Peters, S.L., and Alewijnse, A.E. (2007). Sphingosine-1-phosphate signaling in the cardiovascular system. Curr. Opin. Pharmacol. *7*, 186-192.

Reiter, E., and Lefkowitz, R.J. (2006). GRKs and beta-arrestins: roles in receptor silencing, trafficking and signaling. Trends Endocrinol. Metab. *17*, 159-165.

Rivera, J., Proia, R.L., and Olivera, A. (2008). The alliance of sphingosine-1-phosphate and its receptors in immunity. Nat. Rev. Immunol. *8*, 753-763.

Rosen, H., Gonzalez-Cabrera, P.J., Sanna, M.G., and Brown, S. (2009). Sphingosine 1-phosphate receptor signaling. Annu. Rev. Biochem. *78*, 743-768.

Rosenbaum, D.M., Rasmussen, S.G., and Kobilka, B.K. (2009). The structure and function of G-protein-coupled receptors. Nature *459*, 356-363.

Rutherford, C., Ord-Shrimpton, F.U., Sands, W.A., Pediani, J.D., Benovic, J.L., McGrath, J.C., and Palmer, T.M. (2005). Phosphorylation-independent internalisation and desensitisation of the human sphingosine-1-phosphate receptor $S1P_3$. Cell. Signal. *17*, 997-1009.

Sanna, M.G., Liao, J., Jo, E., Alfonso, C., Ahn, M.Y., Peterson, M.S., Webb, B., Lefebvre, S., Chun, J., Gray, N., et al. (2004). Sphingosine 1-phosphate (S1P) receptor subtypes $S1P_1$ and $S1P_3$, respectively, regulate lymphocyte recirculation and heart rate. J. Biol. Chem. *279*, 13839-13848.

Sanna, M.G., Wang, S.K., Gonzalez-Cabrera, P.J., Don, A., Marsolais, D., Matheu, M.P., Wei, S.H., Parker, I., Jo, E., Cheng, W.C., et al. (2006) Enhancement of capillary leakage and restoration of lymphocyte egress by a chiral $S1P_1$ antagonist *in vivo*. Nat. Chem. Biol. *2*, 434-441.

Takuwa, Y., Okamoto, Y., Yoshioka, K., and Takuwa, N. (2008).

Sphingosine-1-phosphate signaling and biological activities in the cardiovascular system. Biochim. Biophys. Acta *1781*, 483-488.

Tao, R., Hoover, H.E., Zhang, J., Honbo, N., Alano, C.C., and Karliner, J.S. (2009). Cardiomyocyte $S1P_1$ receptor-mediated extracellular signal-related kinase signaling and desensitization. J. Cardiovasc. Pharmacol. *53*, 486-494.

Watterson, K.R., Johnston, E., Chalmers, C., Pronin, A., Cook, S.J., Benovic, J.L., and Palmer, T.M. (2002). Dual regulation of EDG1/S1P(1) receptor phosphorylation and internalization by protein kinase C and G-protein-coupled receptor kinase 2. J. Biol. Chem. *277*, 5767-5777.

Xin, C., Ren, S., Pfeilschifter, J., and Huwiler, A. (2004) Heterologous desensitization of the sphingosine-1-phosphate receptors by purinoceptor activation in renal mesangial cells. Br. J. Pharmacol. *143*, 581-589.