Sphingosine-1-phosphate receptors : tools and molecular pharmacology

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SPHINGOSINE-1-PHOSPHATE RECEPTORS: TOOLS AND MOLECULAR PHARMACOLOGY

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Cover: a red figured kylix, showing Oedipus sitting before the Sphinx of Thebes c. 470 B.C, inv. no. 16541, Museo Gregoriano Etrusco. Photo made by M.Sarri was used with permission of the Vatican Museum.

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SPHINGOSINE-1-PHOSPHATE RECEPTORS: TOOLS AND MOLECULAR PHARMACOLOGY

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"Traveler, there is no path.
Paths are made by walking"
(Antonio Machade, Spanish poet, 1875-1939)

Abbreviations

aa	amino acid
AA	arachidonic acid
AC	adenylyl cyclase
ADP	adenosine diphosphate
ATP	adenosine triphosphate
$[Ca^{2+}]_i$	intracellular calcium
cAMP	cyclic adenosine monophosphate
CFP	cyan fluorescent protein
CHO	Chinese hamster ovary
DAG	diacyl glycerol
DNA	desoxyribonucleic acid
EC	endothelial cell
ECL	electrogenerated chemi-luminescence
EDG	endothelial differentiation gene
EDTA	ethylenediaminetetraacetic acid
EF-1	elongation factor-1
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
FCS	foetal calf serum
GDP	guanine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GIRK	G-protein inwardly recetifying K^+
GPCR	G-protein coupled receptor
GRK	G-protein coupled receptor kinase
GTP	guanine triphosphate
HBSS	Hank's balanced salt solution
HEK	human embryonic kidney
HRP	horse radish peroxidase
HUVEC	human umbilical vein endothelial cell
IBMX	3-isobutyl-1-methylxanthine
IGF	insulin-like growth factor
IP_3	inositol 1,4,5-trisphosphate

Jnk	c-Jun NH_2 terminal kinase
LDL	low density lipoprotein
LPA	lysophosphatidic acid
MAPK	mitogen-activated protein kinase
MEK	mitogen extracellular kinase
NO	nitric oxide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PhS1P	phytosphingosine 1-phosphate
PI3K	phosphatidylinositol 3-kinase
PIP2	phosphatidylinositol biphosphate
PKC	protein kinase C
PLC	phospholipase C β
PTX	pertussis toxin
RAEC	rat aortic endothelial cell
RFU	relative fluorescence units
RGS	regulators of G-protein signaling
Rho	ras homology
RNA	ribonucleic acid
ROCK	rho-associated kinase
S1P	sphingosine-1-phosphate
SM	sphingomyelin
TFMPP	m-trifluoromethylphenylpiperazine
UTP	uridine 5'-triphosphate
VSMC	vascular smooth muscle cell

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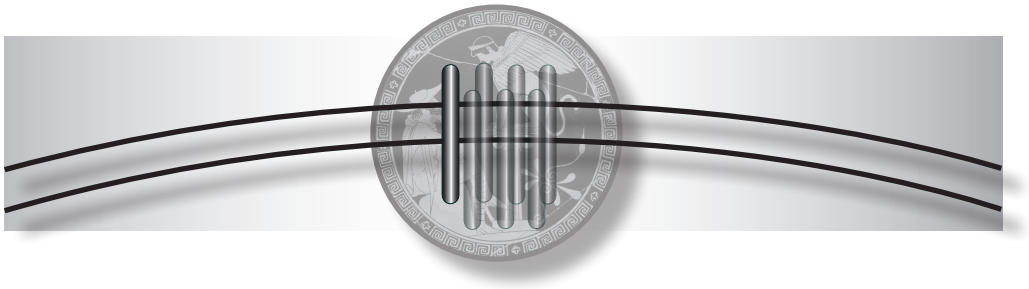
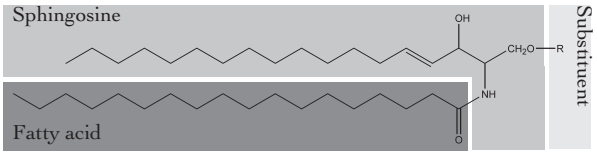


General introduction

"What animal is that which
in the morning goes on four feet,
at noon on two,
and in the evening upon three?"

Introduction to sphingolipids

In 1884, the physician and chemist Johann Ludwig Wilhelm Thudichum (1829-1901) discovered two lipids in the brain characterized by a common aliphatic amino alcohol, for which he used the term "sphingo" (sphinx-like) (Thudichum, 1884) (Figure 1).



Substituent (R)	Sphingolipid name
H	Ceramide
Phosphocholine	Sphingomyelin

Figure 1. Johann Ludwig Wilhelm Thudichum (1829–1901) and the general sphingolipid structure.

In Greek mythology, the sphinx is a demon of death, destruction and bad luck, depicted as a female with the breast, paws and claws of a lion, sometimes with a snake-tail and eagle or gryphon wings. Sitting on a high rock near Thebes, she would post a riddle to all who came before her: "What animal is that which in the morning goes on four feet, at noon on two, and in the evening upon three?" Anyone who could not correctly answer the riddle, would be strangled by her ("sphingo" in Greek means "to strangle"). Until one day Oedipus came along and answered: "Man, who in childhood creeps on hands and knees, in manhood walks erect, and in old age with the aid of a staff." Frustrated that her riddle was solved, the sphinx threw herself off the rock and perished. The Thebans were so grateful to Oedipus for ridding them of the monstrous sphinx, that they made him their king.

Sphingosine-backbone containing lipids (later named sphingolipids by Herb Carter (Carter et al., 1947)) were to Thudichum a part of the dangerous riddle of the brain, similar to the riddle posed by the sphinx. It was already clear at that time that sphingolipids had many different effects, but it still took several decades to solve just parts of their riddle.

Metabolism of sphingolipids

Sphingolipids are a class of lipids representing more than 1000 structurally related compounds in total. At first, sphingolipids like sphingomyelin (SM) and glycosphingolipids were regarded as metabolically inactive building blocks of membranes. In 1989, this idea was challenged when upon extracellular stimulation, sphingomyelinase was able to produce ceramide and phosphorylcholine from sphingomyelin (Okazaki et al., 1989). This was quickly supported by many groups which led to the general believe that this pathway, designated the "SM cycle" or "SM-ceramide pathway", resulted in the release of ceramide as a second messenger. Ceramide could play a role in lipid-mediated signal transduction and influence important processes like apoptosis and cell-cycle arrest (Igarashi, 1997). Nowadays it is known that in this pathway (Figure 2), ceramide can be enzymatically transformed into sphingosine, which is adenosine triphosphate (ATP)-dependently phosphorylated by sphingosine kinase 1 or 2 (SphK1 or SphK2) into sphingosine-1-phosphate (S1P) (Le Stunff et al., 2002; Spiegel & Milstien, 2003; Tani et al., 2007). S1P can be irreversibly degraded to hexadecenal and phosphoethanolamine by S1P lyase (SPL) (Le Stunff et al., 2002; Spiegel & Milstien, 2003; Tani et al., 2007). In addition, S1P can be dephosphorylated either by S1P phosphohydrolase (SPP) or by membrane-associated lipid phosphate phosphohydrolases of which three forms excist (Le Stunff et al., 2002; Spiegel & Milstien, 2003; Tani et al., 2007).

Discovery of sphingolipid receptors

In 1990, Hla and Maciag published the discovery of a transcript which was induced in differentiating human endothelial cells (Hla & Maciag, 1990). Based upon sequence homology with other genes it became evident that this transcript encoded for a protein that belonged to the family of G-protein coupled receptors (GPCRs). Since the ligand for this receptor was unknown, they termed this orphan receptor endothelial differentiation gene 1 (EDG)-1 (Hla & Maciag, 1990). In the following years, several other genes were identified based upon homology to EDG-1 (Okazaki et al., 1993; Masana et al., 1995; Yamaguchi et al., 1996; An et al., 1998; Gräler et al., 1998; Glickman et al., 1999; Im et al., 2000(a); Im et al., 2000(b)), making up a family of in total eight orphan GPCRs, named EDG-1 through EDG-8. Research intensified and resulted eight years after the discovery of EDG-1 in the identification by two independent groups simultaneously of S1P as the ligand for the EDG-1 receptor (Lee et al., 1998; Zondag et al., 1998).

Four additional receptors of the eight-member EDG receptor group turned out to be potently activated by S1P (namely EDG-3,5,6 and 8), while the other three (EDG-2,4 and 7) were activated by a different phospholipid: lyso-phosphatidic acid (LPA). Subsequently, consistent with IUPHAR guidelines, the EDG receptors with high affinity for S1P were renamed to $S1P_{1-5}$ receptors in chronological order of their discovery, the ones being activated by LPA to LPA_{1-3} receptors (Chun et al., 2002). Beside the first group of EDG receptors, three more GPCRs have been reported to have high affinity for S1P: GPR3, GPR6 and GPR12 (Uhlenbrock et al., 2002), but it remains to be confirmed whether they indeed are S1P receptors (Alewijnse & Michel, 2006). Recently, also two more LPA receptors have been identified, named LPA_4 (previously GPR23) (Noguchi et al., 2003) and LPA_5 (previously GPR92) (Lee et al., 2006).

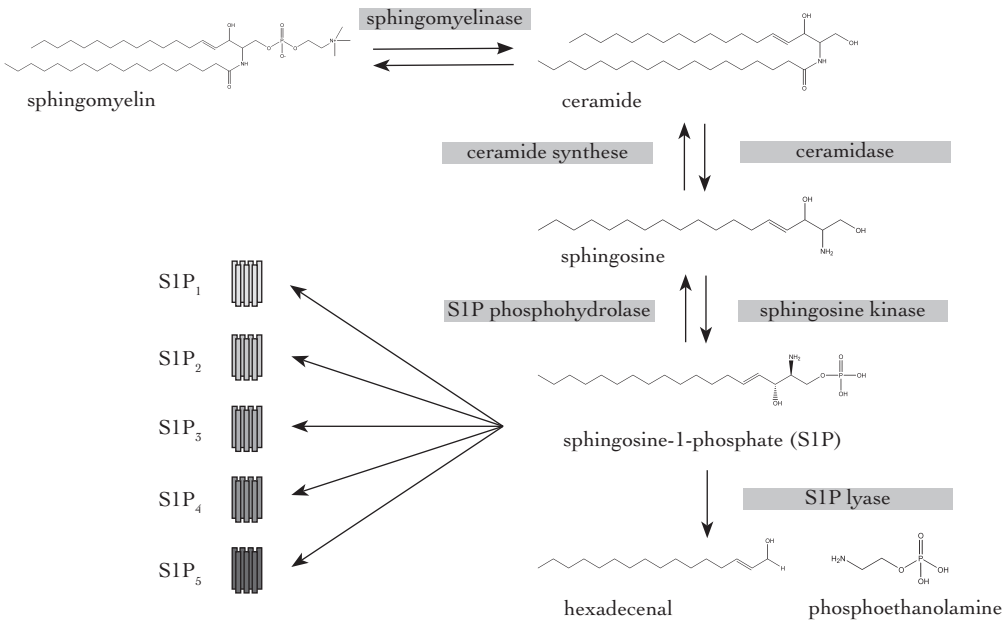


Figure 2. Sphingolipid metabolism.

G-protein coupled receptors and their signaling

As mentioned before, the S1P receptors belong to the GPCR family, which is the largest family of membrane receptors. These receptors couple to intracellular effector systems via the activation of G-proteins thereby transducing signals from the outside to inside of the cell (for a review on GPCRs, see Kristiansen, 2004). GPCRs generally consist of an extracellular N-terminus, an intracellular C-terminus

and seven hydrophobic transmembrane domains connected by hydrophilic loops (Figure 3). Activation of GPCRs accounts for approximately 80% of the signals induced by hormones or neurotransmitters (Birnbaumer et al., 1990). GPCRs are divided into seven classes: Class A (Rhodopsin like), Class B (Secretin like), Class C (Metabotropic glutamate/pheromone), Class D (Fungal pheromone), Class E (cAMP receptors), Ocular albinism proteins and the Frizzled/Smoothened family. S1P receptors belong to the first class, the class A GPCRs.

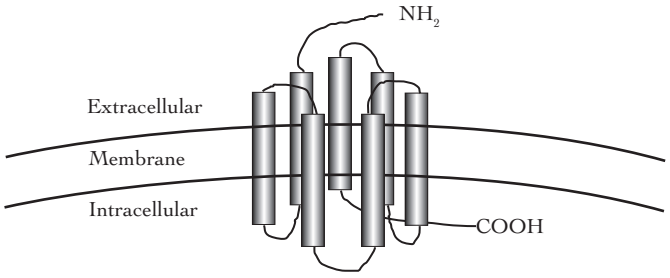


Figure 3. Schematic structure of a GPCR.

As the name already indicates, activation of GPCRs subsequently results in coupling to and activation of heterotrimeric G-proteins which consist of an α-subunit bound to a βγ-dimer (for a review on heterotrimeric G-proteins see Carbrera-Vera et al., 2003). To date, 28 α, 6 β and 12 γ-subunits have been described (Hermans, 2003; Carbrera-Vera et al., 2003) opening the possibility of a multitude of different combinations. G-proteins are divided into 4 subfamilies, based on the sequence similarity of the α-subunit: G_s (G_s and G_{olf}), G_i (G_{tr}, G_{tc}, G_g, G_{i1-3}, G_o and G_z), G_q (G_q, G_{11}, G_{14} and $G_{15/16}$) and G_{12} (G_{12} and G_{13}). In general, the G_s-subfamily activates adenylyl cyclase (AC) subtype 1-9 (Pierce et al., 2002), resulting in increased levels of cAMP whereas the G_i-subfamily does the opposite by inhibiting activation of AC1, AC5 and AC6 thereby decreasing the formation of cAMP (Kamenetsky et al., 2006). The βγ-subunits of the G_i-subfamily can, dependent upon the subtype of AC, either activate (AC2, AC4 and AC7) or inhibit (AC1) adenylyl cyclase. In addition, βγ-subunits can activate many more effectors, amongst others phosphatidylinositol 3-kinase (PI3K) (Pierce et al., 2002), G-protein coupled receptor kinase (GRK)1,2,4, ATP-sensitive K^+-channels, Ca^{2+}-channels, small GTP binding proteins (rho and rac).

$G_{o,}$ which belongs to the same subfamily as G_i is involved in the inhibition of Ca^{2+}-channels and the activation of G-protein inwardly rectifying K^+ (GIRK)-channels. The G_q-subfamily of G-proteins activates phospholipase C (PLC) $\beta_{1\text{-}4}$ (Pierce et al., 2002), which leads to the hydrolysis of phosphatidylinositol biphosphate (PIP2) resulting in the formation of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG subsequently activates protein kinase C (PKC). IP_3 regulates increases in intracellular calcium ($[Ca^{2+}]_i$). The G_{12}-subfamily finally regulates ras homology (rho) guanine nucleotide exchange factors (rhoGEFs) (Jiang et al., 1998, Pierce et al., 2002).

GPCR signaling can generally be initiated by molecules, also referred to as agonists, which induce a receptor conformation that increases the affinity of the receptor for the G-protein. In the inactive state, the α-subunit of the heterotrimeric G-protein is bound to guanine diphosphate (GDP), but upon binding by a guanine nucleotide exchange factors (GEFs) such as a agonist-activated receptor, the GDP is rapidly exchanged for guanine triphosphate GTP (Figure 4). This will lead to a reduced affinity of the α-subunit for the βγ-dimer and cause dissociation of the α-subunit from the βγ-dimer.

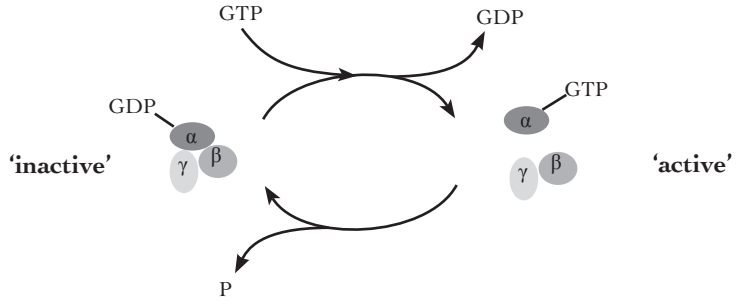


Figure 4. The G-protein activation/inactivation cycle.

For G-proteins belonging to the G_i-subfamily, dissociation of the trimer is blocked by pertussis toxin (PTX), a toxin produced by the bacterium *Bordetella pertussis*. It is the A-subunit of this toxin that is functional in inactivating the G_i-protein by catalysing adenosine diphosphate (ADP) ribosylation of the GDP bound α-subunit. Because of this ability to inactivate G_i (Wong & Rosoff, 1996), PTX has become a widely used pharmacological tool to investigate GPCR signaling (Wong & Rosoff, 1996). After dissociation, both α and βγ-subunits will diffuse through the membrane

to activate or inhibit a large variety of effector proteins as already described above thereby affecting many cellular effects. GPCR signaling is terminated when the GTP bound to the α-subunit is hydrolyzed to GDP by the intrinsic GTPase activity of the α-subunit. The resulting α-GDP will dissociate from the effector and re-unite with the βγ-complex to complete the G-protein activation/inactivation cycle. The intrinsic GTPase activity of the α-subunit increases upon coupling to an effector protein but it can also be increased by GTPase-activating proteins such as regulators of G-protein signaling (RGS) proteins (for a review on RGS proteins see Willars, 2006). The RGS proteins thus on one hand accelerate the termination of GPCR signaling while these proteins on the other hand increase the amount of G-proteins available for signaling.

As can be concluded from the description above, compounds that do not change the affinity of the receptor for the G-protein will not initiate signaling. Some of these compounds are also referred to as antagonists if they can antagonize the effects of agonists by occupying the binding-site on the receptor. In the mid-1990s it became clear that GPCRs can exhibit constitutive activity meaning that they can spontaneously adopt conformations that are able to bind to and activate G-proteins (for review see Bond & IJzerman, 2006). This phenomenon led to a general reclassification of ligands as some of these ligands were shown to decrease the constitutive receptor activity thereby acting as inverse agonists, whereas others, that did not affect the constitutive activity, were classified as neutral antagonists.

Originally GPCRs were considered to selectively activate only one specific family of G-proteins. It has now been shown that GPCRs can couple to and activate different G-protein subtypes simultaneously (Woodside, 2002). Momentarily, it is still not fully understood what determines the G-protein specificity of GPCRs. Mutagenesis studies have revealed certain critical domains in the intracellular loops and/or the C-terminus of a GPCR to be important for coupling to and activation of specific G-proteins (Moro et al., 1993; Parent et al., 1996; Havlickova et al., 2002; Gilchrist et al., 2002; Lawson & Wheatley, 2004; Kostenis et al., 2005). However, this has not yet revealed one conserved sequence which determines binding to G_s , G_i , and/or $G_{q/11}$-proteins specifically. In this context, one domain which as been investigated by many groups is the highly conserved D/ERY motif, situated at the border of the 3rd transmembrane domain and the 2nd intracellular loop (Figure 5). This region contains a triplet of amino acids, Asp/Glu-Arg-Tyr. Conservation of these residues is 86 % for the Asp/Glu residue, 97 % for the Arg residue and 67 % for the Tyr

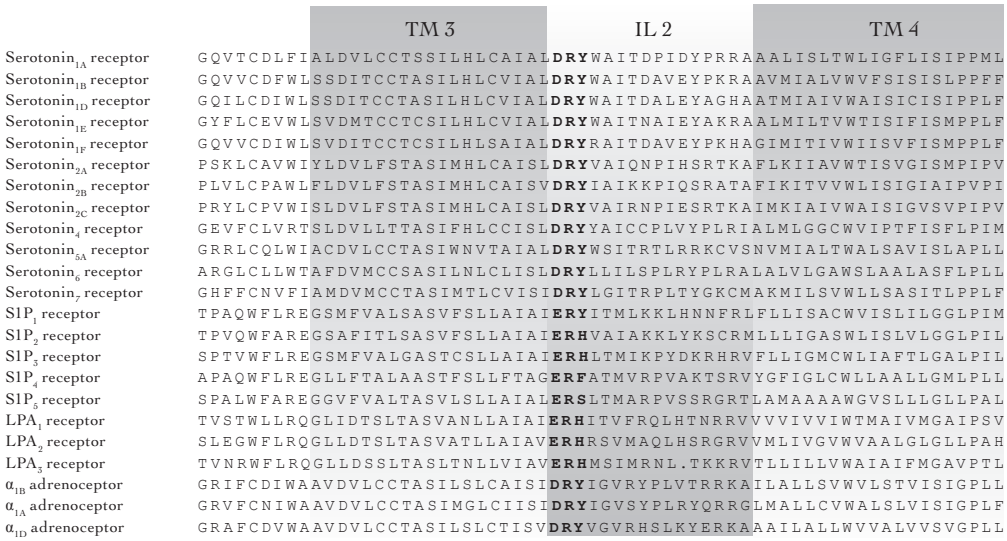


Figure 5. Alignment of the TM3 and IL2 of a selection of Class A GPCRs. The bold letters represent the highly conserved D/ERY motif. Borders of the transmembrane domains (TM) and intracellular loops (IL) are indicated on top.

residue respectively for class A GPCRs (Mirzadegan et al., 2003). Several studies have been performed to investigate the involvement of this domain on G-protein coupling (Ohyama et al., 2002; Capra et al., 2004; Favre et al., 2005; Rovati et al., 2007). Although, this motif is generally believed to play an important role in G-protein coupling and/or activation, the effects of mutations in this motif are not consistent and seem to differ among GPCRs, even within a certain class. For class A GPCRs this has already led to the idea that the receptors belonging to this group can probably be divided into two groups that make use of this motif in a different way (Capra et al., 2004). For one group including e.g. the α_{1B}-adrenergic receptor (Scheer et al., 2000), the β_2-adrenergic receptor (Rasmussen et al., 1999), the histamine H_2 receptor (Alewijnse et al., 2000) and the vasopressin type II receptor (Morin et al., 1998), this motif is involved in keeping the receptor in an inactive ground state. For this group of receptors, mutations in this motif generally retain G-protein binding and increase or induce constitutive receptor activity which is in some cases accompanied by an increase in agonist affinity (Rovati et al., 2007). In the second group, including e.g. the α_{2A}-adrenergic receptor, the M_1 muscarinic acetylcholine receptor and the thromboxane $A_{2\alpha}$ receptor, mutations in the D/ERY motif result in changes in agonist-induced responses, without inducing constitutive receptor activity. So far, many regions in the GPCR structure have been found to be involved in specificity of G-protein coupling, but no overall domain or region has been identified yet.

Since GPCRs can couple to and activate different subtypes of G-proteins simultaneously, activation of a receptor by an agonist may result in the activation of more than one signal transduction pathway. In classical pharmacology, it was generally believed that activation of a receptor by an agonist always produces the same effect, regardless of which agonist is used. The effect can however change in intensity, measured by efficacy or potency. It is now becoming clear that this idea is out of date. This means that a ligand can be a full agonist on one pathway but only a partial agonist or even an antagonist for another pathway (Hermans, 2003) (Figure 6).

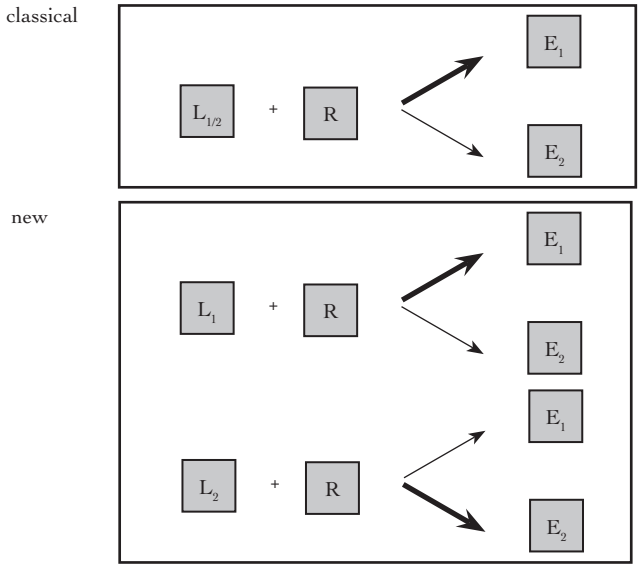


Figure 6. Schematic view of classical pharmacology and ligand-directed signaling (L = Ligand, R = Receptor, E = Effect).

A very illustrative example of ligand-directed signalling comes from the serotonin field. Here it has been shown that M-trifluoromethylphenylpiperazine (TFMPP) and bufotenin, both believed to be full agonists for the 5-HT2 serotonin receptor, indeed had differential effects on different pathways (Stout et al., 2002). TFMPP was a full agonist at IP accumulation but had only partial intrinsic activity at arachidonic acid (AA) release. In contrast, bufotenin was a full agonist at AA release and only a partial agonist at IP accumulation (Stout et al., 2002). Many names for this concept are used currently, for example agonist-specific trafficking, signaling-selective agonism, functional selectivity and ligand-directed signaling (Perez & Karnik, 2005; Urban et al., 2007). Since there is no concensus yet, we will name this concept ligand-directed signaling.

Desensitization of GPCR signaling

When a GPCR is subject to long-term ligand exposure, the risk of over-stimulation exists. This is prevented by a process called desensitization, resulting in a lower response to the same level of agonist exposure and/or a need to increase the concentration of agonist to maintain the same response (for a review, see Pierce et al., 2002). Desensitization can involve many processes and mechanisms, some of which directly affect receptor responsiveness and can be either homologous or heterologous. In the first case, desensitization is a result of activation of the receptor itself, while heterologous desensitization is caused by activation of other receptors or stimuli. One mechanism of desensitization starts with the recruitment of GRKs to an activated receptor. The GRKs subsequently phosphorylate the receptor, thereby enabling the binding of arrestins. To date, six GRK isoforms are identified ($GRK_{1\text{-}6}$) which are all activated by protein kinases, like PKC or c-Src (Pronin & Benovic, 1997; Sarnago et al., 1999). Beside GRKs, other kinases like PKC or mitogen-activated protein kinase (MAPK) can also phospohorylate the receptor (Diviani et al., 1997; Smith et al., 1998). Phosphorylation of the receptor and binding of arrestins to the receptor will uncouple the receptor from the G-protein thereby terminating signaling. A second mechanism important in desensitization of GPCR signaling involves internalization of the receptor, which can be arrestin-dependent or independent (Bünemann & Hosey, 1999). The GPCR is transported from the cell membrane into clathrin-coated pits from where they can be either recycled to the membrane or be degraded, in which case the pits are called lysozomes. The described mechanisms are all rapid desensitization, while there is also long-term desensitization. This involves receptor down-regulation via increased degradation and/or reduced de novo synthesis.

S1P receptor signaling

The signaling pathways of S1P receptors have been extensively investigated and summarized in several excellent reviews (Pyne & Pyne, 2001; Kluk & Hla, 2002; Sanchez & Hla, 2004; Anliker & Chun, 2004 (a&b); Ishii et al., 2004; Young & Van Brocklyn, 2006; Meyer zu Heringdorf & Jakobs, 2007). An overview of the signaling of S1P receptors is described here and schematically depicted in figure 7.

• The $S1P_1$ receptor is ubiquitously expressed throughout the body with the highest overall expression in the brain (brain > lung ≈ spleen > heart/vasculature > kidney) (Chae et al., 2004(b)). The $S1P_1$ receptor is a unique member of the S1P receptor family as it is the only receptor which selectively couples to a single G-protein family, i.e. G_i-proteins (Lee et al., 1996; Ancellin & Hla, 1999; Windh et al., 1999), to inhibit AC and subsequently decrease cAMP formation (Zondag et al., 1998; Van Brocklyn et al., 1998; Okamoto et al., 1998; Kon et al., 1999).

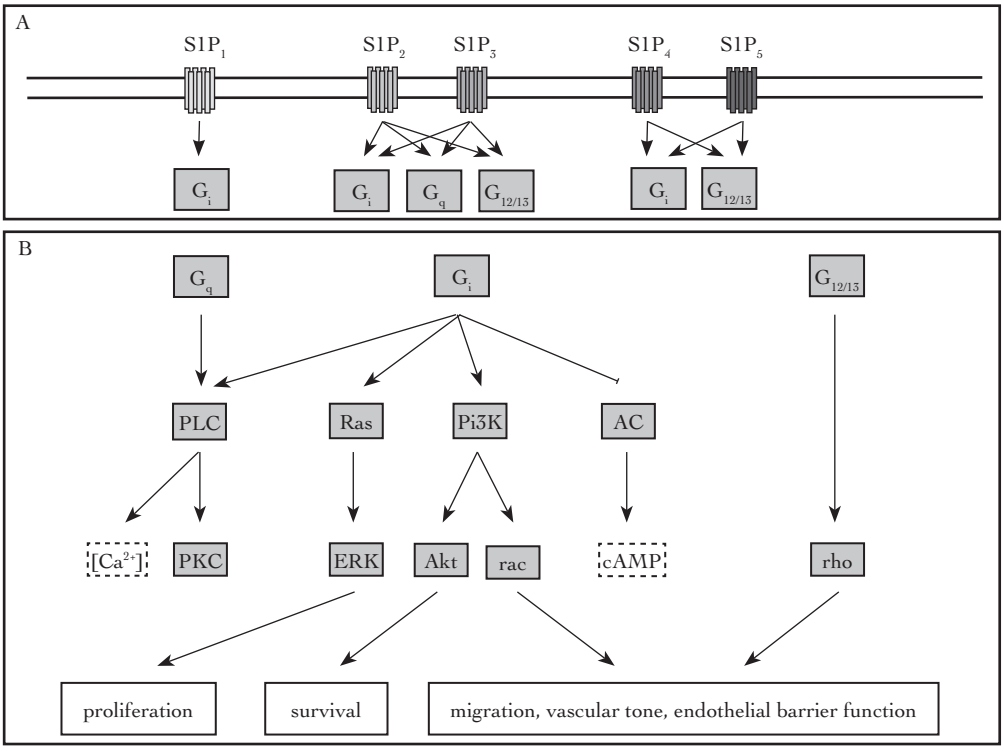


Figure 7. G-protein coupling of S1P receptors (A) and the subsequent signaling pathways of the specific G-proteins (B). (PLC: phospholipase C; Pi3K: phosphatidylinositol 3-kinase; AC: adenylyl cyclase; PKC: protein kinase C; ERK: extracellular signal-regulated kinase).

The $S1P_1$ receptor has also been shown to activate ERK-1/-2 in a PTX-dependent manner in various systems (Lee et al., 1996; Lee et al., 1998; Zondag et al., 1998; Okamoto et al., 1998; Okamoto et al., 1999). Small, G_i-dependent increases in intracellular calcium ($[Ca^{2+}]_i$) levels have also been observed, most likely via the activation of phospholipase C β (PLC) by the βγ-subunit of G_i-proteins, since the $S1P_1$ receptor does not couple to G_q-proteins (Zondag et al., 1998, Okamoto et al., 1998; Van Brocklyn et al., 1998; Kon et al., 1999, Ancellin & Hla, 1999; Lee et al., 1999; Okamoto et al., 1999, An et al., 1999). Support for the activation of

PLC via the βγ-subunit of G_i is the finding that preincubation with U73122 (a PLC inhibitor) or overexpression of $G_{\alpha t}$ (a βγ-subunit scavenger) can indeed block the $S1P_1$-mediated $[Ca^{2+}]_i$ increases (Okamoto et al., 1998). However, conflicting results were reported with regard to whether these $[Ca^{2+}]_i$ increases are inceed PLC mediated (Kon et al., 1999; Okamoto et al., 1999). Recently, experiments in smooth muscle cells using silencing of $S1P_1$ or specific activation of the $S1P_1$ receptor with the agonist SEW2871 showed that the $S1P_1$ receptor does not activate PLCβ (Hu et al., 2006 (b)). With regard to the small GTPases rho and rac, the $S1P_1$ receptor can activate rac, but the data on rho are inconclusive (Kluk & Hla, 2002). In human umbilical vein endothelial cells (HUVECs), activation of rho has been shown (Lee et al., 1998; Paik et al., 2001) whereas in smooth muscle cells the $S1P_1$-mediated activation of rho was not confirmed (Hu et al., 2006(b)). In addition, Chinese hamster ovary (CHO) cells either showed no effect (Okamoto et al., 2000) or an activation of rho by the $S1P_1$ receptor (Paik et al., 2001).

- Expression of the $S1P_2$ receptor is like the $S1P_1$ receptor also seen in many different cell types where it can activate G_i, G_q and $G_{12/13}$-proteins (Ancellin & Hla, 1999; Windh et al., 1999; An et al., 1999). Via activation of G_q-proteins, stimulation of the $S1P_2$ receptor leads to increases in $[Ca^{2+}]_i$ via activation of PLC and subsequent IP_3 formation (Kon et al., 1999; Ancellin & Hla., 1999 An et al., 1999; Gonda et al., 1999; Kupperman et al., 1999). The $S1P_2$ receptor can also activate ERK-1/-2, c-Jun NH_2-terminal kinase (Jnk) and p38 MAPK (An et al., 1997; Okamoto et al., 1999; Gonda et al., 1999; Kupperman et al., 2000; An et al., 2000). It has recently been shown that the combination of $S1P_2$ and $S1P_3$ receptor activation is required for activation of Akt (Means et al., 2007). The $S1P_2$ receptor can activate one of the small GTPases, namely rho (Okamoto et al., 2000; Gonda et al., 1999), but it can inhibit the activation of rac and lower its basal activity (Okamoto et al., 2000). This most likely occurs via the activation of phosphatase and tensin homolog (PTEN), which antagonizes the PI3-Akt pathways, resulting in a down-regulation of rac and Akt activity (Sanchez et al., 2007). Although the $S1P_2$ receptor through G_i can regulate AC (Kon et al., 1999; Gonda et al., 1999) it seems to increase rather than decrease cAMP levels in CHO cells. Interestingly, these studies suggest that this is not caused via the activation of G_s (Windh et al., 1999) and also not via $[Ca^{2+}]_i$ (Kon et al., 1999). Probably, $S1P_2$ receptor activation leads to an inhibition of phosphodiesterase activity, the enzymes which normally break down cAMP, consequently resulting in an increase in cAMP levels.

- The $S1P_3$ receptor is expressed in the same tissues as mentioned for the $S1P_1$ and $S1P_2$ receptor with minor differences in expression level. Like the $S1P_2$ receptor, it couples to G_i, G_q and $G_{12/13}$-proteins (Ancellin & Hla, 1999; Windh et al., 1999). Activation of the $S1P_3$ receptor regulates AC. However, this effect has been shown to be either an inhibition in a PTX-dependent manner or a stimulation of AC (Kon et al., 1999; Okamoto et al., 1999). The $S1P_3$ receptor is also linked to the $PLC/IP_3/[Ca^{2+}]_i$ pathway (Kon et al., 1999; Okamoto et al., 1999; An et al., 1999; Ishii et al., 2001), suggested to be even more efficiently than the $S1P_2$ receptor (Ancellin et al., 1999). The increase in $[Ca^{2+}]_i$ has been shown to be affected both by G_i and G_q-coupled pathways (Sato et al., 1999). Activation of the MAPK cascade has been shown via detection of ERK-1/-2 phosphorylation and c-Jun mRNA and protein expression (An et al., 1997; Okamoto et al., 1999; An et al., 2000). $S1P_3$ activation also results in activation of rho and rac (Okamoto et al., 2000; Liu et al., 2000; Paik et al., 2001). In mouse embryo fibroblasts however, no activation of rac was measured upon S1P stimulation (Ishii et al., 2001).
- The $S1P_4$ receptor shows a very restricted expression pattern, being found only in the lung (Jolly et al., 2002) and lymphoid tissues (Gräler et al., 1998). The affinity of S1P for the $S1P_4$ receptor is lower than for the other four receptors, whereas the affinity for phyto-S1P is higher. It couples to G_i and $G_{12/13}$-proteins, but not to G_s or G_q-proteins (Gräler et al., 2003). S1P-stimulation of the $S1P_4$ receptor results in activation of ERK-1\-2, PLC and elevations of $[Ca^{2+}]_i$ (Van Brocklyn et al., 2000; Yamazaki et al., 2000). PLC is activated through the G_i-pathway since it is blocked by PTX treatment (Gräler et al., 2003). The $S1P_4$ receptor can activate rho, but the effect on rac still has to be determined (Gräler et al., 2003).
- The $S1P_5$ receptor expression is limited to the white matter of the brain (Im et al., 2000(b); Terai et al., 2003), skin and spleen (Im et al., 2000(b); Ishii et al., 2001). The $S1P_5$ receptor activates G_i and $G_{12/13}$, but not G_s and G_q-proteins (Im et al., 2000(b); Malek et al., 2001). Activation of the $S1P_5$ receptor inhibits AC, which is PTX-sensitive (Im et al., 2000(b); Malek et al., 2001). Interestingly, the inhibition of AC is already evident in the absence of S1P, suggesting constitutive activity of this receptor subtype (Niedernberg et al., 2003). In this way, inhibition of ERK activity, but not rho occurs (Niedernberg et al., 2003). $S1P_5$ can also activate Jnk and inhibit ERK-1/-2 activation, but not via G_i-activation (Malek et al., 2001). It has been shown that via G_i, $S1P_5$ can activate PI3K and it can activate rho kinase via $G_{12/13}$ (Li et al., 2003; Novgorodov et al., 2007). $S1P_5$ can also activate Akt (Jaillard et al., 2005)

Important structures in S1P receptors

Among GPCRs, entire motifs, single amino acids or general properties of amino acids have been conserved (Figure 5). This indicates their importance in specific receptor functions, e.g. ligand binding or G-protein coupling. 3D structures of many GPCRs, including the S1P receptors are currently only computational predictions from the DNA sequence, based upon the crystal structure of the bovine rhodopsin receptor provided by Palczewski et al. in 2000. These predicted structures can provide information about important amino acids for binding the ligand, and important domains for coupling to G-proteins.

For the S1P receptors, most research has been done on the $S1P_1$ receptor, which revealed some residues that are critical for interaction with its ligand, S1P (Fujiwara et al., 2007). Based upon these predictions, mutant receptors can be made and studied for verification the models. These studies indeed confirmed some important amino acids for interaction with S1P. Arg^{120} and Arg^{292} form two ion pairs with the anionic amine group of S1P, while Glu^{121} forms one ion pair with the amine group of S1P (Parrill et al., 2000). These sites are also predicted to be important for interaction with SEW2871, which strengthens the prediction (Fujiwara et al., 2007). Another important region for S1P binding are 2 tyrosine residues in the N-terminal tail of the $S1P_1$ receptor. It has been shown that O-sulfation of these two amino acids is required for high-affinity S1P binding (Fieger et al., 2005). This seems to be a $S1P_1$ receptor specific feature, since the other S1P receptors do not contain a tyrosine with the required neighboring aspartic acid and only the $S1P_2$ receptor contains a tyrosine flanked by a glutamic acid, but this is not a site for sulfation. For the $S1P_4$ receptor, Trp^{291}, Glu^{284} and Thr^{127} are important for S1P binding according to computational predictions (Vaidehi et al., 2002). This has been opposed by a model by Holdsworth et al. in 2004 since all mentioned residues are directed towards extracellular loops, and are thus unlikely to interact with S1P. They show an important function of Glu^{122} which once mutated to Gln switches the $S1P_4$ receptor from S1P to LPA responsiveness (Holdsworth et al., 2004).

A very interesting motif in S1P receptors which has not yet been studied is the D/ERY motif. A striking difference can be found when comparing this motif to the G-protein coupling of these receptors. The $S1P_1$ receptor, which only couples to $G_{i/o}$-proteins, contains an ERY motif whereas the other S1P receptor subtypes contain an ERH/S/F motif and couple to G_q, $G_{12/13}$-proteins ($S1P_{4,5}$) and to G_i-proteins ($S1P_{2,3}$) (Figure 7).

Desensitization of S1P receptors

Desensitization of S1P receptors has not been that intensively studied yet, even though it seems to be the functional mechanism of a new drug, FTY720 (Matloubian et al., 2004). Shortly after S1P was found to be the ligand for the EDG-1 receptor, a paper appeared which showed S1P-induced trafficking of this receptor, C-terminally tagged with GFP (Liu et al., 1999). S1P was able to internalize the EDG-1-GFP receptor and after S1P wash out, the receptor was recycled to the membrane. Internalization of a C-terminal GFP-tagged $S1P_1$ receptor was also seen after insulin-like growth factor (IGF)-1 and IGF-2 stimulation, which could be blocked by dimethylsphingosine, showing the involvement of sphingosine kinase (El-Shewy et al., 2006). A loss of sensitivity to S1P has been shown after 10 minutes of S1P or FTY720-P (the phosphorylated, active form of FTY720) stimulation of renal mesangial cells (Xin et al., 2004). This loss of sensitivity could also be induced heterologously by stimulation with ATP, UTP or adenosine. When ATP or UTP were used, increased cAMP levels, PKC activation and another staurosporine-sensitive protein kinase were involved. The $S1P_1$ receptor internalized to endocytic vesicles and lysosomes from which it could be recycled to the membrane (Liu et al., 1999). Internalization of the receptor required GRK2 activation and subsequently two different pathways have been shown for two different ligands (Oo et al., 2007). The $S1P_1$ receptor will be recycled to the membrane after S1P-induced internalization, but will be broken down after FTY720-P-induced internalization (Oo et al., 2007). This has been ascribed to rapid polyubiquitinylation of the $S1P_1$ receptor by FTY720-P, but not by S1P (Oo et al., 2007). In contrast to the $S1P_1$ receptor, the $S1P_3$ receptor was shown to internalize without being phosphorylated (Rutherford et al., 2005).

S1P signaling in the vasculature

The two major cell types in the vasculature are endothelial cells (ECs) and vascular smooth muscle cells (VSMCs). The $S1P_{1-3}$ receptors are differentially expressed on each cell type (Michel et al., 2007) (Table 1). Interestingly, since both cell types express all enzymes necessary to produce S1P (Yatomi et al., 2001), S1P can be formed locally in the vessel wall by endothelial as well as vascular smooth muscle cells (Yatomi et al., 2001; Alewijnse et al., 2004). However, S1P is also a major constituent in blood and plasma in concentrations of 200-900 nM (Murata et al., 2000). It can be released from activated platelets (Yatomi et al., 2000) and is a

major constituent of erythrocytes (Pappu et al., 2007), so it may also reach vascular cells via the bloodstream. S1P is a constituent of HDL (Murata et al., 2000), but it is unknown if it can activate its receptors in the vessel wall in this form. Most of the plasma S1P is bound to proteins and hence unlikely to be available for receptor interaction. In blood, the free S1P concentration is unknown but most likely lower than in plasma. The effect of S1P on both ECs and VSMCs and the total vessel is described below and depicted in figure 8.

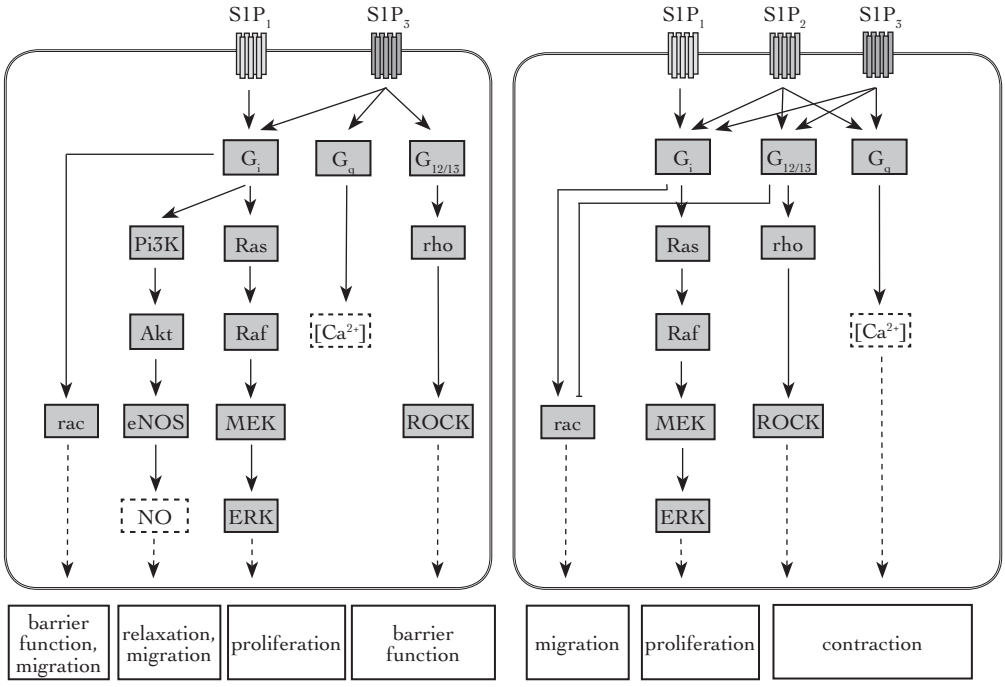


Figure 8. S1P receptor signaling in endothelial cells (left panel) and vascular smooth muscle cells (right panel) (Pi3K: phosphatidylinositol 3-kinase; eNOS: endothelial nitric oxide synthase; NO: nitric oxide; ERK: extracellular signal-regulated kinase; ROCK: rho-associated kinase; MEK: mitogen extracellular kinase).

Endothelial cells

The $S1P_1$ and $S1P_3$ receptors are most abundantly expressed in the endothelium (Panetti, 2002) (Table 1). On ECs, one of the effects of S1P is maintaining barrier function. Although the underlying mechanism is not clear yet, it involves activation of the small GTPase rac (McVerry & Garcia, 2004). Barrier function requires reorganization of actin which occurs via Tiam1/rac1 activation via $S1P_1$ (Singleton et al., 2005). The $S1P_3$ receptor seems to have the opposite effect, thus decreasing rather than increasing barrier function (Singleton et al., 2006). Besides

the effect on barrier function, S1P also causes migration of ECs via rearrangement of the cytoskeleton through activation of rac1. Activation of rho is also involved in migration of ECs. Inhibition of PI3-K decreases migration induced by S1P (Tamama & Okajima, 2002), but this does not affect S1P-mediated rac1 activation (Tamama & Okajima, 2002; Gonzalez et al., 2006). S1P-induced activation of PI3-K can also lead to the activation of endothelial nitric oxide synthase (eNOS) through Akt, resulting in the production of the vasodilator NO and subsequent relaxation of a vessel. A final effect of S1P on ECs is proliferation in a G_i-sensitive way via both $S1P_1$ and $S1P_3$ receptors. Rho activation plays a key role in this process, whereas ERK activation is not involved (Tamama & Okajima, 2002; Panetti, 2002).

Vascular smooth muscle cells

VSMCs generally express high levels of the $S1P_2$ receptor compared to the $S1P_1$ and $S1P_3$ receptor (Table 1). S1P affects migration of VSMCs, which is stimulated via $S1P_1$ and $S1P_3$ receptor activation, but inhibited via $S1P_2$ receptor activation (Waeber et al., 2004). Migration occurs via the activation or inhibition of rac1. The effect of S1P on VSMC migration is thus depending on the S1P receptor expression profile. S1P can also induce proliferation of VSMCs via all three receptors. In case of $S1P_1$ activation, the PI3-K pathway is involved. Through S1P-mediated activation of G_q, $[Ca^{2+}]_i$ will rise via the PLC pathway, which results in contraction of the vessel.

Vascular tone

The effect of S1P on vascular tone has been investigated by several groups, but conflicting results have been found, possibly due to differences in species, methods, vascular bed, vessel function and S1P concentrations used. S1P-induced constriction has mainly been found in smaller vessels (Bischoff et al., 2000(a); Bischoff et al., 2000(b); Sugiyama et al., 2000; Bischoff et al., 2001; Tosaka et al., 2001; Coussin et al., 2002; Ohmori et al., 2003; Salomone et al., 2003; Hedemann et al., 2004; Czyborra et al., 2006), while relaxation was observed in both smaller and larger vessels (Mogami et al., 1999; Dantas et al., 2003; Tölle et al., 2005; Roviezzo et al., 2006).

The effect of S1P on vascular tone is most likely the result of a balance between the effects on ECs and VSMCs. This balance may amongst others depend on the relative expression of the $S1P_{1\text{-}3}$ receptors on both cell types.

Table 1. Vascular S1P receptor expression in the various cell types present in these vessels.

Whole artery			
	Rat	$S1P_1$, $S1P_2$, $S1P_3$, $S1P_5$	Salomone et al., 2003
	Gerbil	S1P1, $S1P_2$, $S1P_3$	Scherer et al., 2006
	Human	$S1P_1 \approx S1P_3 > S1P_2$	Mazurais et al., 2002
Arterial Smooth Muscle			
	Mouse	$S1P_1$	Chae et al., 2004b
	Rat	$S1P_2 > S1P_3 > S1P_1$; $S1P_4$ absent	Tamama et al., 2001
		$S1P_2 > S1P_3$; $S1P_1$, $S1P_4$ and $S1P_5$ absent	Ryu et al., 2002
		$S1P_2 \approx S1P_3 > S1P_1$	Coussin et al., 2002
		$S1P_3$; $S1P_4$ and $S1P_5$ absent	Yamaguchi et al., 2003
	Human	$S1P_2 > S1P_3 > S1P_1$	Tamama et al., 2001
		$S1P_3$; $S1P_2$ in aorta but not coronary; $S1P_1$ absent	Mazurais et al., 2002
		$S1P_2 > S1P_3$; $S1P_1$, $S1P_4$ and $S1P_5$ absent	Ryu et al., 2002
		$S1P_2 > S1P_1 > S1P_3 > S1P_5$; S1P4 absent	Damirin et al., 2005
Arterail endothelium			
	Mouse	$S1P_1$	Chae et al., 2004
	Rat	$S1P_1 > S1P_3$; $S1P_2$ absent	Tamama et al., 2001
	Bovine	S1P1 > $S1P_3 > S1P_2$	Wang et al., 1999
	Human	$S1P_1 > S1P_3 > S1P_2$	Tamama et al., 2001
		$S1P_1$ and $S1P_3$; $S1P_2$ absent	Kimura et al., 2000
		$S1P_1$; $S1P_3$ absent	Mazurais et al., 2002
Venous smooth muscle			
	Human	$S1P_1$	Hla & Maciag, 1990
Venous endothelium			
	Human	$S1P_1$	Hla & Maciag, 1990
		$S1P_1 > S1P_3$; $S1P_2$ absent	Lee et al., 1999
		$S1P_1 > S1P_3 > S1P_2$	Wang et al., 1999
		$S1P_1 > S1P_3$; $S1P_2$, $S1P_4$ and $S1P_5$ absent	Ryu et al., 2002

S1P receptors in vascular disease

Receptors that are important in development and/or functioning of any tissue are often also involved in diseases. This also holds true for the S1P receptors. Even though S1P can mediate wound healing for instance in the vessel by stimulating angiogenesis, it can become atherogenic and thrombogenic and influence cardiovascular diseases (Gardell et al., 2006). S1P is not only involved in vessel formation during the embryonic state, as was found in studies using $S1P_1$ receptor knockout mice, but also after birth. Therefore, S1P will have an effect on diseases in which angiogenesis (new vessel formation) plays a role, such as cancer, psoriasis and rheumatoid arthritis (Pyne & Pyne, 2000).

S1P also influences proliferation, which plays a role in atherosclerosis. In this disease, thrombus formation is induced by S1P released from thrombin-stimulated platelets (Gardell et al., 2006). Deposition of Ox-LDL in the thrombus results in activation of sphingomyelinase and production of S1P (Augé et al., 1999). S1P causes a decrease of the endothelial barrier permeability via G_i-protein activation through the $S1P_1$ and $S1P_3$ receptors. (Siess, 2002; McVerry & Garcia, 2004). S1P also stimulates migration and proliferation of smooth muscle cells into the atherosclerotic plaques, resulting in the occlusion of the vessel. More effects of S1P through its receptors are an acute and transient decrease in heart rate through activation of GIRK channels (Guo et al., 1999; Forrest et al., 2004; Karliner, 2004), decreases coronary artery blood flow (Ohmori et al., 2003; Karliner, 2004) and an initial increase in blood pressure in whole mice through increases of $[Ca^{2+}]_i$ due to release of Ca^{2+} from internal stores, followed by cytoskeletal re-arrangement (Forrest et al., 2004). The opposing effects of S1P on blood pressure make it hard to make a clear statement whether S1P is beneficial or has a negative effect on blood pressure.

Tools to study S1P receptors and their signaling

Knockout models

There are many ways to study the physiological role of newly discovered receptors. One of them is to generate receptor-null mice, also called knockout mice. The first report on S1P receptor knockout mice came in 2000, where Liu et al. described the essence of the $S1P_1$ receptor in vascular maturation (Liu et al., 2000). Disruption of the corresponding gene resulted in lethality at embryonic day E12.5 to E14.5. The cause was found to be a defective vascular maturation, since the VSMCs failed

to surround the nascent endothelial tubes even though they were in the vicinity. Conditional $S1P_1$ receptor knockouts in EC or VSMC showed that vessel coverage by VSMCs is controlled by the $S1P_1$ receptor in ECs (Allende et al., 2003).

Double or triple S1P receptor knockout mice indicated that besides the $S1P_1$ receptor, also the $S1P_2$ and $S1P_3$ receptor play an important role in vascular development (Kono et al., 2004). Even though the $S1P_2$ or $S1P_3$ single knockout mice were viable, and the $S1P_1$/$S1P_3$ double knockout did not alter the phenotype of the $S1P_1$ single null embryo, knocking out the $S1P_2$ receptor together with the $S1P_1$ receptor increased the severity of the phenotype of the single $S1P_1$ receptor knockout (Kono et al., 2004). These embryos died two days earlier in their embryonic phase than the $S1P_1$ single knockout embryos due to bleeding. The triple receptor knockout showed a similar or slightly more severe result than the double knockout (Kono et al., 2004). This indicated that during embryonic angiogenesis, these receptors function coordinately. The $S1P_2$ receptor single knockout mice had spontaneous seizures during 3-7 weeks of age, which occasionally led to death (Yang et al., 2002). The $S1P_3$ receptor single knockout mice had no obvious phenotype (Yang et al., 2002).

Besides vascular defects, $S1P_1$ knockout embryos also show abnormal limb development, which was caused by a loss of $S1P_1$ in endothelial cells (Chae et al., 2004 (a)). Although $S1P_2$ knockout mice do not have an obvious phenotype, they appear to have lower mesenteric and renal resistance (Lorenz et al., 2007). Another effect of knocking out the $S1P_2$ receptor was recently shown by Kono et al. in 2007, who found that these mice were deaf by one month of age, possibly due to vascular defects in the stria vascularis.

The $S1P_3$ null mice were successfully used to show that the observed slower heart rate (bradycardia) after administration of FTY720 was mediated via the $S1P_3$ receptor. Additionally, S1P receptor agonist-induced lymphopenia (inhibition of lymphocyte recirculation) was not altered in these mice, indicating that the $S1P_3$ receptor is most likely not involved in this process (Sanna et al., 2004).

siRNA

Short interfering (si) RNA is a technique in which a piece of a double stranded (ds) RNA fragment is brought into the cell via for example a lentiviral system. Inside the cell, the dsRNA is cleaved by a dicer resulting in siRNA pieces of 21-23 nucleotides. A single strand is incorporated into the RNA-induced silencing complex which can detect and destroy the target mRNA, thereby interrupting protein synthesis.

In case of S1P receptors, this relatively new method has been applied to study receptor function by specific knockdown of a receptor. In endothelial cells the importance of the $S1P_1$ receptor for actin reorganization was shown and also that the $S1P_1$ receptor may protect these cells from necrosis (Krump-Konvalinkova et al., 2005). Another study showed that together with the $S1P_3$ receptor, the $S1P_1$ receptor mediates the inflammatory responses in these cells (Lin et al., 2007). In vascular smooth muscle cells, siRNA proved that contraction is mediated exclusively via $S1P_2$ receptors (Hu et al., 2006(a)). For the $S1P_5$ receptor, usage of this method showed that in oligo-dendrocyte precursos cells migration was inhibited via activation of rho and ROCK through $G_{\alpha 12/13}$ (Novgorodov et al., 2007).

Tagged receptors

When binding studies are unavailable, receptors can be identified by using a molecularly modified receptor, e.g. in receptor regulation studies. It is possible to add a tag to the receptor, e.g. HA-tag or green fluorescent protein (GFP), which is easily detectable by fluorescence microscopy or Western blot for example. As described before using tagged receptors, S1P has been shown to internalize the $S1P_1$ and $S1P_3$ receptor. When different ligands became available, it was also shown that receptor fate is dependent on which ligand is used. $S1P_1$-GFP internalized after stimulation with the $S1P_1$ receptor selective agonist SEW2871 and S1P will be recycled to the membrane, where FTY720-P stimulation leads to internalization followed by degradation (Jo et al., 2005). The same group showed later that this degradation was caused by poly-ubiquitination of the receptor, induced by one agonist, but not by other (Gonzalez-Cabrera et al., 2007).

It is important to realize that adding large tags to receptors can influence signaling and/or regulation of the receptor. This is illustrated in a study on the $S1P_4$ receptor where several different constructs were made. Addition of a C-terminal GFP tag resulted in no membrane localization of the receptor in contrast to their $S1P_1$-GFP receptor. The same group also constructed a N-terminally FLAG-tagged $S1P_4$

receptor, which again did not show membrane localization. Interestingly, a $S1P_4$ receptor with both tags was expressed at the cell surface. The $S1P_4$ receptor with a C-terminal FLAG tag did come to the cells surface, in contrast to the N-terminally FLAG tagged receptor. The same discrepancy was seen when a HA tag (N or C-terminal) was used instead of the FLAG tag. Subsequent experiments showed that S1P induces cell migration through the membrane expressed $S1P_4$ receptor (Kohno et al., 2003).

Specific antibodies

Antibodies directed against a specific receptor or against a ligand may be used to investigate receptor localization, visualize endogenous receptors or even to study regulation of receptors and/or receptor signaling. A $S1P_1$ receptor monoclonal antibody has been described which binds to the human and/or mouse $S1P_1$ receptor in membranes (Goetzl et al., 2004). Binding to the other S1P receptor subtypes has not been studied. It does not inhibit binding of S1P to the receptor, but it inhibits T cell chemotactic responses and suppression of T cell chemotaxis to chemokines, both induced by S1P. Interestingly, another monoclonal antibody, against S1P itself has been shown to reduce tumor progression or even eliminate tumors (Visentin et al., 2006). It also blocks EC migration, which is involved in blood vessel formation and was able to stop the protective effect of S1P against apoptosis of tumor cells. This could well indicate that S1P itself is a potential target in cancer therapy.
On the receptor side, not many antibodies exist and most of them that do exist are not very specific.

Transfection studies

An often used method to investigate receptor signaling are stable cell lines overexpressing receptors. Since they overexpress one specific receptor, signaling differences between the original cells and the stable cells are then assigned to the overexpressed receptor. A better control than using the original cells is using mock transfected cells. Mock transfected cells are made the same way as the stable cells, but are transfected with only the empty vector (plasmid) and not the plasmid containing the target gene DNA. This then rules out possible effects of the plasmid like antibiotic resistance.
A disadvantage of overexpression systems is the possibility that receptors will activate G-proteins they would normally not activate in native cells. Another thing

to keep in mind when interpreting the results obtained with stable cell lines is endogenous (native) receptors. Even when overexpressing the receptor of interest, the endogenous receptors are still present and functional in the cells. They can thus still cause part of the observed effect(s). Therefore it is preferable to create stable cell lines from cells that have no or as little as possible receptors expressed that have affinity for the used ligand.

The most used cells in S1P receptor research are CHO, human embryonic kidney (HEK) 293 cells and HUVECs. However, endogenous S1P receptor expression of all of these cells is not very well investigated yet. The few reports that do exist show that in CHO-K1 cells, endogenous mRNA expression of the $S1P_1$, $S1P_2$ and $S1P_4$ receptor was detected (Holdsworth et al., 2005). In HEK293 cells, no endogenous expression of the $S1P_1$ receptor was found (Lee et al., 1998). The primary cell line HUVECs, like other ECs (see above), most likely express the $S1P_1$ and $S1P_3$ receptor, but this is based on functional data, not on direct measurements of receptor presence itself (Butler et al., 2004).

Ligands

Pharmacological S1P receptor studies have been hampered due to a scarce availability of ligands (Figure 9). The few that are available are described below.

Agonists: The novel immunosuppressive drug FTY720, a chemical modification of myriocin (ISP-1) from the fungus *Isaria sinclairii*, already was described in 1996 (Suzuki et al., 1996) but it was only in 2002 when it was found to exert its effect via S1P receptors (Brinkmann et al., 2002). They showed that only the phosphorylated form (FTY720-P), which resembles the structure of S1P, activates $S1P_{1,3-5}$ receptors. Recently, clinical trials using FTY720 revealed an effect on heart rate. Administration of this compound induced bradycardia in healthy subjects (Schmouder et al., 2006). This FTY720-induced reduction in heart rate was also shown in mice, but was absent in $S1P_3$ receptor knockout mice (Forrest, 2004). VPC23153 and VPC24191 are synthetic agonists for the $S1P_1$ and $S1P_3$ receptor. Pytosphingosine 1-phosphate (PhS1P) was found as an agonist to $S1P_4$, with much higher affinity than for the $S1P_5$ receptor (Candelore et al., 2002). The first report on the $S1P_1$ selective agonist SEW2871 appeared in 2005 (Bolick et al., 2005).

Antagonists: The first S1P receptor antagonist was suramin, an antagonist for the $S1P_3$ receptor, but suramin also blocks other receptor ligand interactions (Ancellin & Hla, 1999) and has receptor-independent effects on G-protein function

(Freissmuth et al., 1996). SB649146, a $S1P_1$ receptor inverse agonist attenuated the S1P-induced activation of p42/p44 MAPK in HEK293 cells (Waters et al., 2006). There was no effect of this compound on GTPγS binding in membranes expressing the $S1P_2$, $S1P_3$ or LPA_1 receptor. VPC23019 is a synthetic antagonist for the $S1P_1$ and $S1P_3$ receptor. A specific $S1P_2$ receptor antagonist, JTE-013 was described in 2002 (Osada et al., 2002). A new $S1P_3$ antagonist, BML-241, was found upon a search for compounds that inhibit S1P-induced rises in $[Ca^{2+}]_i$ for 37% at 10 μM (Koide et al., 2002). Recently, a specific $S1P_1$ receptor antagonist, named W146, was described (Sanna et al., 2004).

A common problem with newly found or developed ligands is that they are usually poorly characterized. It is therefore important that compounds are investigated thoroughly before usage.

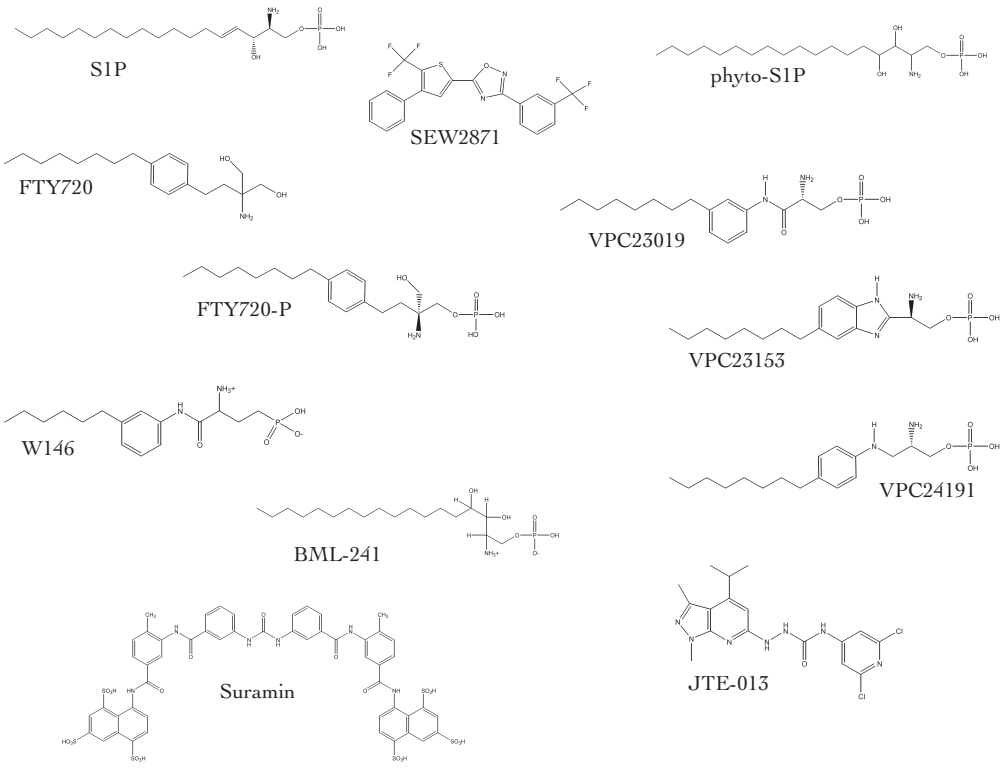


Figure 9. Chemical structures of several S1P receptor ligands.

Aim of the project

When one aims to study GPCRs, many different tools are needed. In the relatively new field of S1P receptors, these tools are not overly present. The few that do exist are usually poorly characterized due to their short existence. Therefore, the first part of this thesis was aimed at characterizing and/or improving existing tools and to develop new ones. This part focussed on characterizing one specific new antagonist by studying its effect on various signaling pathways of S1P receptors and other receptors (chapter 2), and on setting up a new method to study the internalization mechanisms of the S1P receptors in more detail (chapter 3).

The second part of this project focused on the application of the available or newly developed tools to characterize the S1P receptors. Our newly developed immunocytochemical method was applied to compare the internalization of these receptors (chapter 5) and to discover if the D/ERY motif was involved in this process. The latter by also looking at the effect of mutations in this motif on G_i or G_q-coupled pathways for the $S1P_{1-3}$ receptors (chapter 6). The many new ligands that are being developed open a pharmacological way to study certain effects induced via S1P receptors. They were used to assign effects of S1P receptor ligands in the rat aorta to S1P receptor subtypes expressed in endothelial or smooth muscle cells (chapter 4). These ligands also need to be thouroughly tested, which we did by investigating their effects on the multiple signaling pathways activated via the $S1P_3$ receptor (chapter 7).

References

Alewijnse AE, Timmerman H, Jacobs EH, Smit MJ, Roovers E, Cotecchia S, Leurs R (2000). The effect of mutations in the DRY motif on the constitutive activity and structural instability of the histamine H(2) receptor. *Mol Pharmacol* **57**:890-898

Alewijnse AE, Peters SL, Michel MC (2004). Cardiovascular effects of sphingosine-1-phosphate and other sphingomyelin metabolites. *Br J Pharmacol* **143**:666–684

Alewijnse AE, Michel MC (2006). Sphingosine-1-phosphate and sphingosylphosphorylcholine: two of a kind? *Br J Pharmacol* **147**:347-348

Allende ML, Yamashita T, Proia RL (2003). G-protein-coupled receptor S1P1 acts within endothelial cells to regulate vascular maturation. *Blood* **102**:3665-3667

An S, Bleu T, Huang W, Hallmark OG, Coughlin SR, Goetzl EJ (1997). Identification of cDNAs encoding two G protein-coupled receptors for lysosphingolipids. *FEBS Lett* **417**:279-282

An S, Bleu T, Hallmark OG, Goetzl EJ (1998). Characterization of a novel subtype of human G protein-coupled receptor for lysophosphatidic acid. *J Biol Chem* **273**:7906-7910

An S, Bleu T, Zheng Y (1999). Transduction of intracellular calcium signals through G protein-mediated activation of phospholipase C by recombinant sphingosine 1-phosphate receptors. *Mol Pharmacol*. **55**:787-794

An S, Zheng Y, Bleu T (2000). Sphingosine 1-phosphate-induced cell proliferation, survival, and related signaling events mediated by G protein-coupled receptors Edg3 and Edg5. *J Biol Chem* **275**:288-96.

Ancellin N, Hla T (1999). Differential pharmacological properties and signal transduction of the sphingosine 1-phosphate receptors EDG-1, EDG-3, and EDG-5. *J Biol Chem* **274**:18997-19002

Anliker B, Chun J (2004(a)). Cell surface receptors in lysophospholipid signaling. *Semin Cell Dev Biol* **15**:457-465

Anliker B, Chun J (2004(b)). Lysophospholipid G protein-coupled receptors. *J Biol Chem* **279**:20555-20558

Augé N, Nikolova-Karakashian M, Carpentier S, Parthasarathy S, Nègre-Salvayre A, Salvayre R, Merrill AH, Levade T (1999). Role of sphingosine 1-phosphate in the mitogenesis induced by oxidized low density lipoprotein in smooth muscle cells via activation of sphingomyelinase, ceramidase, and sphingosine kinase. *J Biol Chem* **274**:21533-21538

Awad AS, Ye H, Huang L, Li L, Foss FW, Macdonald TL, Lynch KR, Okusa MD (2006). Selective sphingosine 1-phosphate 1 receptor activation reduces ischemia-reperfusion injury in mouse kidney. *Am J Physiol Renal Physiol* **290**:F1516-1524

Birnbaumer L, Abramowitz J, Brown AM (1990). Receptor-effector coupling by G proteins. *Biochim Biophys Acta* **1031**:163-224

Bischoff A, Czyborra P, Fetscher C, Meyer Zu Heringdorf D, Jakobs KH, Michel MC (2000(a)). Sphingosine-1-phosphate and sphingosylphosphorylcholine constrict renal and mesenteric microvessels in vitro. *Br J Pharmacol* **130**:1871-1877

Bischoff A, Czyborra P, Meyer Zu Heringdorf D, Jakobs KH, Michel MC (2000(b)). Sphingosine-1-phosphate reduces rat renal and mesenteric blood flow in vivo in a pertussis toxin-sensitive manner. *Br J Pharmacol* **130**:1878-1883

Bischoff A, Finger J, Michel MC (2001). Nifedipine inhibits sphinogosine-1-phosphate-induced renovascular contraction in vitro and in vivo. *Naunyn Schmiedebergs Arch Pharmacol* **364**:179-182

Bolick DT, Srinivasan S, Kim KW, Hatley ME, Clemens JJ, Whetzel A, Ferger N, Macdonald TL, Davis MD, Tsao PS, Lynch KR, Hedrick CC (2005). Sphingosine-1-phosphate prevents tumor necrosis factor-{alpha}-mediated monocyte adhesion to aortic endothelium in mice. *Arterioscler Thromb Vasc Biol* **25**:976-981

Bond RA, IJzerman AP (2006). Recent developments in constitutive receptor activity and inverse agonism, and their potential for GPCR drug discovery. *Trends Pharmacol Sci* **27**:92-96

Brinkmann V, Davis MD, Heise CE, Albert R, Cottens S, Hof R, Bruns C, Prieschl E, Baumruker T, Hiestand P, Foster CA, Zollinger M, Lynch KR (2002). The immune modulator FTY720 targets sphingosine 1-phosphate receptors. *J Biol Chem* **277**:21453-21457

Bünemann M, Hosey MM (1999). G-protein coupled receptor kinases as modulators of G-protein signaling. *J Physiol* **517**: 5-23

Butler J, Lana D, Round O, LaMontagne K (2004). Functional characterization of sphingosine 1-phosphate receptor agonist in human endothelial cells. *Prostaglandins Other Lipid Mediat* **73**:29-45

Cabrera-Vera TM, Vanhauwe J, Thomas TO, Medkova M, Preininger A, Mazzoni MR, Hamm HE (2003). Insights into G protein structure, function, and regulation. *Endocr Rev* **24**:765-781

Candelore MR, Wright MJ, Tota LM, Milligan J, Shei GJ, Bergstrom JD, Mandala SM (2002). Phytosphingosine 1-phosphate: a high affinity ligand for the S1P(4)/Edg-6 receptor. *Biochem Biophys Res Commun* **297**:600-606

Capra V, Veltri A, Foglia C, Crimaldi L, Habib A, Parenti M, Rovati GE (2004). Mutational analysis of the highly conserved ERY motif of the thromboxane A2 receptor: alternative role in G protein-coupled receptor signaling. *Mol Pharmacol* **66**:880-889

Carter HE, Haines WJ, Ledyard WE, Norris WP (1947). Biochemistry of the sphingolipids. I. preparation of sphingolipids from beef brain and spinal cord. *J Biol Chem* **169**:77-82

Chae SS, Paik JH, Allende ML, Proia RL, Hla T (2004(a)). Regulation of limb development by the sphingosine 1-phosphate receptor S1P1/EDG-1 occurs via the hypoxia/VEGF axis. *Dev Biol* **268**:441-447

Chae SS, Proia RL, Hla T (2004(b)). Constitutive expression of the S1P1 receptor in adult tissues. *Prostaglandins Other Lipid Mediat* **73**:141-150
Coussin F, Scott RH, Wise A, Nixon GF (2002). Comparison of sphingosine 1-phosphate-induced intracellular signaling pathways in vascular smooth muscles: differential role in vasoconstriction. *Circ Res* **91**:151-157
Chun J, Goetzl EJ, Hla T, Igarashi Y, Lynch KR, Moolenaar W, Pyne S, Tigyi G (2002). International Union of Pharmacology. XXXIV. Lysophospholipid receptor nomenclature. *Pharmacol Rev* **54**:265-269
Czyborra C, Bischoff A, Michel MC (2006). Indomethacin differentiates the renal effects of sphingosine-1-phosphate and sphingosylphosphorylcholine. *Naunyn Schmiedebergs Arch Pharmacol* **373**:37-44
Damirin A, Tomura H, Komachi M, Tobo M, Sato K, Mogi C, Nochi H, Tamoto K, Okajima F (2005). Sphingosine 1-phosphate receptors mediate the lipidinduced cAMP accumulation through cyclooxygenase-2/prostaglandin I2 pathway in human coronary artery smooth muscle cells. *Mol Pharmacol* **67**:1177–1185
Dantas AP, Igarashi J, Michel T (2003). Sphingosine 1-phosphate and control of vascular tone. *Am J Physiol Heart Circ Physiol* **284**:H2045-2052
Diviani D, Lattion AL, Cotecchia S (1997). Characterization of the phosphorylation sites involved in G protein-coupled receptor kinase- and protein kinase C-mediated desensitization of the alpha1B-adrenergic receptor. *J Biol Chem* **272**:28712-28719
El-Shewy HM, Johnson KR, Lee MH, Jaffa AA, Obeid LM, Luttrell LM (2006). Insulin-like growth factors mediate heterotrimeric G protein-dependent ERK1/2 activation by transactivating sphingosine 1-phosphate receptors. *J Biol Chem* **281**:31399-31407
Favre N, Fanelli F, Missotten M, Nichols A, Wilson J, di Tiani M, Rommel C, Scheer A (2005). The DRY motif as a molecular switch of the human oxytocin receptor. *Biochemistry* **44**:9990-10008
Fieger CB, Huang MC, Van Brocklyn JR, Goetzl EJ (2005). Type 1 sphingosine 1-phosphate G protein-coupled receptor signaling of lymphocyte functions requires sulfation of its extracellular amino-terminal tyrosines. *FASEB J* **19**:1926-1928
Forrest M, Sun SY, Hajdu R, Bergstrom J, Card D, Doherty G, Hale J, Keohane C, Meyers C, Milligan J, Mills S, Nomura N, Rosen H, Rosenbach M, Shei GJ, Singer II, Tian M, West S, White V, Xie J, Proia RL, Mandala S (2004). Immune cell regulation and cardiovascular effects of sphingosine 1-phosphate receptor agonists in rodents are mediated via distinct receptor subtypes. *J Pharmacol Exp Ther* **309**:758-768
Freissmuth M, Boehm S, Beindl W, Nickel P, IJzerman AP, Hohenegger M, Nanoff C (1996). Suramin analogues as subtype-selective G protein inhibitors. *Mol Pharmacol* **49**:602-611
Fujiwara Y, Osborne DA, Walker MD, Wang DA, Bautista DA, Liliom K, Van Brocklyn JR, Parrill AL, Tigyi G (2007). Identification of the hydrophobic ligand binding pocket of the S1P1 receptor. *J Biol Chem* **282**:2374-2385
Gardell SE, Dubin AE, Chun J (2006). Emerging medicinal roles for lysophospholipid signaling. *Trends Mol Med* **12**:65-75
Gilchrist A, Li A, Hamm HE (2002). G alpha COOH-terminal minigene vectors dissect heterotrimeric G protein signaling. *Sci STKE* **2002**:PL1
Glickman M, Malek RL, Kwitek-Black AE, Jacob HJ, Lee NH (1999). Molecular cloning, tissue-specific expression, and chromosomal localization of a novel nerve growth factor-regulated G-protein- coupled receptor, nrg-1. *Mol Cell Neurosci* **14**:141-152
Goetzl EJ, Dembrow D, Van Brocklyn JR, Gräler M, Huang MC (2004). An IgM-kappa rat monoclonal antibody specific for the type 1 sphingosine 1-phosphate G protein-coupled receptor with antagonist and agonist activities. *Immunol Lett* **93**:63-69
Gonda K, Okamoto H, Takuwa N, Yatomi Y, Okazaki H, Sakurai T, Kimura S, Sillard R, Harii K, Takuwa Y (1999). The novel sphingosine 1-phosphate receptor AGR16 is coupled via pertussis toxin-sensitive and -insensitive G-proteins to multiple signalling pathways. *Biochem J* **337**:67-75
Gonzalez E, Kou R, Michel T (2006). Rac1 modulates sphingosine 1-phosphate-mediated activation of phosphoinositide 3-kinase/Akt signaling pathways in vascular endothelial cells. *J Biol Chem* **281**:3210-3216
Gonzalez-Cabrera PJ, Hla T, Rosen H (2007). Mapping pathways downstream of sphingosine 1-phosphate subtype 1 by differential chemical perturbation and proteomics. *J Biol Chem* **282**:7254-7264
Gräler MH, Bernhardt G, Lipp M (1998). EDG6, a novel G-protein-coupled receptor related to receptors for bioactive lysophospholipids, is specifically expressed in lymphoid tissue. *Genomics* **53**:164-169
Gräler MH, Grosse R, Kusch A, Kremmer E, Gudermann T, Lipp M (2003). The sphingosine 1-phosphate receptor S1P4 regulates cell shape and motility via coupling to Gi and G12/13. *J Cell Biochem* **89**:507-519
Guo J, MacDonell KL, Giles WR (1999). Effects of sphingosine 1-phosphate on pacemaker activity in rabbit sino-atrial node cells. *Pflugers Arch* **438**:642-648
Havlickova M, Prezeau L, Duthey B, Bettler B, Pin JP, Blahos J (2002). The intracellular loops of the GB2 subunit are crucial for G-protein coupling of the heteromeric gamma-aminobutyrate B receptor. *Mol Pharmacol* **62**:343-350
Hedemann J, Fetscher C, Michel MC (2004). Comparison of noradrenaline and lysosphingolipid-induced vasoconstriction in mouse and rat small mesenteric arteries. *Auton Autacoid Pharmacol* **24**:77-85
Hermans E (2003). Biochemical and pharmacological control of the multiplicity of coupling at G-protein-coupled receptors. *Pharmacol Ther* **99**:25-44
Hla T, Maciag T (1990). An abundant transcript induced in differentiating human endothelial cells encodes a polypeptide with structural similarities to G-protein-coupled receptors. *J Biol Chem* **265**:9308-9313

Holdsworth G, Osborne DA, Pham TT, Fells JI, Hutchinson G, Milligan G, Parrill AL (2004). A single amino acid determines preference between phospholipids and reveals length restriction for activation of the S1P4 receptor. *BMC Biochem* **5**:12

Holdsworth G, Slocombe P, Hutchinson G, Milligan G (2005). Analysis of endogenous S1P and LPA receptor expression in CHO-K1 cells. *Gene* **350**:59-63

Hu W, Huang J, Mahavadi S, Li F, Murthy KS (2006(a)). Lentiviral siRNA silencing of sphingosine-1-phosphate receptors S1P1 and S1P2 in smooth muscle. *Biochem Biophys Res Commun* **343**:1038-1044

Hu W, Mahavadi S, Huang J, Li F, Murthy KS. Characterization of S1P1 and S1P2 receptor function in smooth muscle by receptor silencing and receptor protection. *Am J Physiol Gastrointest Liver Physiol* **291**:G605-610

Igarashi Y (1997). Functional roles of sphingosine, sphingosine 1-phosphate, and methylsphingosines: in regard to membrane sphingolipid signaling pathways. *J Biochem (Tokyo)* **122**:1080-1087

Im DS, Heise CE, Harding MA, George SR, O'Dowd BF, Theodorescu D, Lynch KR (2000(a)). Molecular cloning and characterization of a lysophosphatidic acid receptor, Edg-7, expressed in prostate. *Mol Pharmacol* **57**:753-759

Im DS, Heise CE, Ancellin N, O'Dowd BF, Shei GJ, Heavens RP, Rigby MR, Hla T, Mandala S, McAllister G, George SR, Lynch KR (2000(b)). Characterization of a novel sphingosine 1-phosphate receptor, Edg-8. *J Biol Chem* **275**:14281-14286

Ishii I, Friedman B, Ye X, Kawamura S, McGiffert C, Contos JJ, Kingsbury MA, Zhang G, Brown JH, Chun J (2001). Selective loss of sphingosine 1-phosphate signaling with no obvious phenotypic abnormality in mice lacking its G protein-coupled receptor, LP(B3)/EDG-3. *J Biol Chem* **276**:33697-33704

Jaillard C, Harrison S, Stankoff B, Aigrot MS, Calver AR, Duddy G, Walsh FS, Pangalos MN, Arimura N, Kaibuchi K, Zalc B, Lubetzki C (2005). Edg8/S1P5: an oligodendroglial receptor with dual function on process retraction and cell survival. *J Neurosci* **25**:1459-1469

Jiang Y, Ma W, Wan Y, Kozasa T, Hattori S, Huang XY (1998). The G protein G alpha12 stimulates Bruton's tyrosine kinase and a rasGAP through a conserved PH/BM domain. *Nature* **395**:808-813

Jo E, Sanna MG, Gonzalez-Cabrera PJ, Thangada S, Tigyi G, Osborne DA, Hla T, Parrill AL, Rosen H (2005). S1P1-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 PS, Rosenfeldt HM, Milstien S, Spiegel S (2002). The roles of sphingosine-1-phosphate in asthma. *Mol Immunol* **38**:1239-1245

Kamenetsky M, Middelhaufe S, Bank EM, Levin LR, Buck J, Steegborn C (2006). Molecular details of cAMP generation in mammalian cells: a tale of two systems. *J Mol Biol* **362**:623-639

Karliner JS (2004). Mechanisms of cardioprotection by lysophospholipids. *J Cell Biochem* **92**:1095-1103

Kimura T, Watanabe T, Sato K, Kon J, Tomura H, Tamama K, Kuwabara A, Kanda T, Kobayashi I, Ohta H, Ui M, Okajima F (2000). Sphingosine 1-phosphate stimulates proliferation and migration of human endothelial cells possibly through the lipid receptors, Edg-1 and Edg-3. *Biochem J* **348**:71–76.

Kluk MJ, Hla T (2002). Signaling of sphingosine-1-phosphate via the S1P/EDG-family of G-protein-coupled receptors. *Biochim Biophys Acta* **1582**:72-80

Kohno T, Matsuyuki H, Inagaki Y, Igarashi Y (2003). Sphingosine 1-phosphate promotes cell migration through the activation of Cdc42 in Edg-6/S1P4-expressing cells. *Genes Cells* **8**:685-697

Koide Y, Hasegawa T, Takahashi A, Endo A, Mochizuki N, Nakagawa M, Nishida A (2002). Development of novel EDG3 antagonists using a 3D database search and their structure-activity relationships. *J Med Chem* **45**:4629-4638

Kon J, Sato K, Watanabe T, Tomura H, Kuwabara A, Kimura T, Tamama K, Ishizuka T, Murata N, Kanda T, Kobayashi I, Ohta H, Ui M, Okajima F (1999). Comparison of intrinsic activities of the putative sphingosine 1-phosphate receptor subtypes to regulate several signaling pathways in their cDNA-transfected Chinese hamster ovary cells. *J Biol Chem* **274**:23940-23947

Kono M, Belyantseva IA, Skoura A, Frolenkov GI, Starost MF, Dreier JL, Lidington D, Bolz SS, Friedman TB, Hla T, Proia RL (2007). Deafness and stria vascularis defects in S1P2 receptor-null mice. *J Biol Chem* **282**:10690-10696

Kono M, Mi Y, Liu Y, Sasaki T, Allende ML, Wu YP, Yamashita T, Proia RL (2004). The sphingosine-1-phosphate receptors S1P1, S1P2, and S1P3 function coordinately during embryonic angiogenesis. *J Biol Chem* **279**:29367-29373

Kostenis E, Martini L, Ellis J, Waldhoer M, Heydorn A, Rosenkilde MM, Norregaard PK, Jorgensen R, Whistler JL, Milligan G (2005). A highly conserved glycine within linker I and the extreme C terminus of G protein alpha subunits interact cooperatively in switching G protein-coupled receptor-to-effector specificity. *J Pharmacol Exp Ther* **313**:78-87

Kristiansen K (2004). Molecular mechanisms of ligand binding, signaling, and regulation within the superfamily of G-protein-coupled receptors: molecular modeling and mutagenesis approaches to receptor structure and function. *Pharmacol Ther* **103**:21-80

Kupperman E, An S, Osborne N, Waldron S, Stainier DY (2000). A sphingosine-1-phosphate receptor regulates cell migration during vertebrate heart development. *Nature* **406**:192-195

Krump-Konvalinkova V, Yasuda S, Rubic T, Makarova N, Mages J, Erl W, Vosseler C, Kirkpatrick CJ, Tigyi G, Siess W (2005). Stable knock-down of the sphingosine 1-phosphate receptor S1P1 influences multiple functions of human endothelial cells. *Arterioscler Thromb Vasc Biol* **25**:546-552

Lawson Z, Wheatley M (2004). The third extracellular loop of G-protein-coupled receptors: more than just a linker between two important transmembrane helices. *Biochem Soc Trans* **32**:1048-1050

Lee CW, Rivera R, Gardell S, Dubin AE, Chun J (2006). GPR92 as a new G12/13- and Gq-coupled lysophosphatidic acid receptor that increases cAMP, LPA5. *J Biol Chem* **281**:23589-23597
Lee MJ, Evans M, Hla T (1996). The inducible G protein-coupled receptor edg-1 signals via the G(i)/mitogen-activated protein kinase pathway. *J Biol Chem* **271**:11272-11279
Lee MJ, Van Brocklyn JR, Thangada S, Liu CH, Hand AR, Menzeleev R, Spiegel S, Hla T (1998). Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. *Science* **279**:1552-1555
Lee MJ, Thangada S, Claffey KP, Ancellin N, Liu CH, Kluk M, Volpi M, Sha'afi RI, Hla T (1999). Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. *Cell* **99**:301-312
Le Stunff H, Peterson C, Liu H, Milstien S, Spiegel S (2002). Sphingosine-1-phosphate and lipid phosphohydrolases. *Biochim Biophys Acta* **1582**:8-17
Li Y, Gonzalez MI, Meinkoth JL, Field J, Kazanietz MG, Tennekoon GI (2003). Lysophosphatidic acid promotes survival and differentiation of rat Schwann cells. *J Biol Chem* **278**:9585-9591
Lin CI, Chen CN, Lin PW, Lee H (2007). Sphingosine 1-phosphate regulates inflammation-related genes in human endothelial cells through S1P1 and S1P3. *Biochem Biophys Res Commun* **355**:895-901
Liu CH, Thangada S, Lee MJ, Van Brocklyn JR, Spiegel S, 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 CX, Hobson JP, Rosenfeldt HM, Nava VE, Chae SS, Lee MJ, Liu CH, Hla T, Spiegel S, Proia RL (2000). Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. *J Clin Invest* **106**:951-961
Lorenz JN, Arend LJ, Robitz R, Paul RJ, MacLennan AJ (2007). Vascular dysfunction in S1P2 sphingosine 1-phosphate receptor knockout mice. *Am J Physiol Regul Integr Comp Physiol* **292**:R440-446
Malek RL, Toman RE, Edsall LC, Wong S, Chiu J, Letterle CA, Van Brocklyn JR, Milstien S, Spiegel S, Lee NH (2001). Nrg-1 belongs to the endothelial differentiation gene family of G protein-coupled sphingosine-1-phosphate receptors. *J Biol Chem* **276**:5692-5699
Masana MI, Brown RC, Pu H, Gurney ME, Dubocovich ML (1995). Cloning and characterization of a new member of the G-protein coupled receptor EDG family. *Receptors Channels* **3**:255-262
Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, Brinkmann V, Allende ML, Proia RL, Cyster JG (2004). Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature* **427**: 355-360
Mazurais D, Robert P, Gout B, Berrebi-Bertrand I, Laville MP, Calmels T (2002). Cell type-specific localization of human cardiac S1P receptors. *J Histochem Cytochem* **50**: 661–670
McVerry BJ, Garcia JG (2004). Endothelial cell barrier regulation by sphingosine 1-phosphate. *J Cell Biochem* **92**:1075-1085
Means CK, Xiao CY, Li Z, Zhang T, Omens JH, Ishii I, Chun J, Brown JH (2007). Sphingosine 1-phosphate S1P2 and S1P3 receptor-mediated Akt activation protects against in vivo myocardial ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol* **292**:H2944-2951
Meyer zu Heringdorf D, Jakobs KH (2007). Lysophospholipid receptors: signalling, pharmacology and regulation by lysophospholipid metabolism. *Biochim Biophys Acta* **1768**:923-940
Michel MC, Mulders AC, Jongsma M, Alewijnse AE, Peters SL (2007). Vascular effects of sphingolipids. *Acta Paediatr Suppl.* **96**:44-48
Mirzadegan T, Benkö G, Filipek S, Palczewski K (2003). Sequence analyses of G-protein-coupled receptors: similarities to rhodopsin. *Biochemistry* **42**:2759-2767
Mogami K, Mizukami Y, Todoroki-Ikeda N, Ohmura M, Yoshida K, Miwa S, Matsuzaki M, Matsuda M, Kobayashi S (1999). Sphingosylphosphorylcholine induces cytosolic Ca(2+) elevation in endothelial cells in situ and causes endothelium-dependent relaxation through nitric oxide production in bovine coronary artery. *FEBS Lett* **457**:375-380
Morin D, Cotte N, Balestre MN, Mouillac B, Manning M, Breton C, Barberis C (1998). The D136A mutation of the V2 vasopressin receptor induces a constitutive activity which permits discrimination between antagonists with partial agonist and inverse agonist activities. *FEBS Lett* **441**:470-475
Moro O, Lameh J, Högger P, Sadée W (1993). Hydrophobic amino acid in the i2 loop plays a key role in receptor-G protein coupling. *J Biol Chem* **268**:22273-22276
Murata N, Sato K, Kon J, Tomura H, Yanagita M, Kuwabara A, Ui M, Okajima F (2000). Interaction of sphingosine 1-phosphate with plasma components, including lipoproteins, regulates the lipid receptor-mediated actions. *Biochem J* **352**:809-815
Niedernberg A, Blaukat A, Schöneberg T, Kostenis E (2003). Regulated and constitutive activation of specific signalling pathways by the human S1P5 receptor. *Br J Pharmacol* **138**:481-493
Noguchi K, Ishii S, Shimizu T (2003). Identification of p2y9/GPR23 as a novel G protein-coupled receptor for lysophosphatidic acid, structurally distant from the Edg family. *J Biol Chem* **278**:25600-25606
Novgorodov AS, El-Alwani M, Bielawski J, Obeid LM, Gudz TI (2007). Activation of sphingosine-1-phosphate receptor S1P5 inhibits oligodendrocyte progenitor migration. *FASEB J* **21**:1503-1514
Ohmori T, Yatomi Y, Osada M, Kazama F, Takafuta T, Ikeda H, Ozaki Y (2003). Sphingosine 1-phosphate induces contraction of coronary artery smooth muscle cells via S1P2. *Cardiovasc Res* **58**:170-177
Ohyama K, Yamano Y, Sano T, Nakagomi Y, Wada M, Inagami T (2002). Role of the conserved DRY motif on G protein activation of rat angiotensin II receptor type 1A. *Biochem Biophys Res Commun* **292**:362-367

Okamoto H, Takuwa N, Gonda K, Okazaki H, Chang K, Yatomi Y, Shigematsu H, Takuwa Y (1998). EDG1 is a functional sphingosine-1-phosphate receptor that is linked via a Gi/o to multiple signaling pathways, including phospholipase C activation, Ca2+ mobilization, Ras-mitogen-activated protein kinase activation, and adenylate cyclase inhibition. *J Biol Chem* **273**:27104-27110

Okamoto H, Takuwa N, Yatomi Y, Gonda K, Shigematsu H, Takuwa Y (1999). EDG3 is a functional receptor specific for sphingosine 1-phosphate and sphingosylphosphorylcholine with signaling characteristics distinct from EDG1 and AGR16. *Biochem Biophys Res Commun* **260**:203-208

Okamoto H, Takuwa N, Yokomizo T, Sugimoto N, Sakurada S, Shigematsu H, Takuwa Y (2000). Inhibitory regulation of Rac activation, membrane ruffling, and cell migration by the G protein-coupled sphingosine-1-phosphate receptor EDG5 but not EDG1 or EDG3. *Mol Cell Biol* **20**:9247-9261

Oo ML, Thangada S, Wu MT, Liu CH, Macdonald TL, Lynch KR, Lin CY, 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

Okazaki T, Bell RM, Hannun YA (1989(. Sphingomyelin turnover induced by vitamin D3 in HL-60 cells. Role in cell differentiation. *J Biol Chem* **264**:19076-19080

Okazaki H, Ishizaka N, Sakurai T, Kurokawa K, Goto K, Kumada M, Takuwa Y (1993). Molecular cloning of a novel putative G protein-coupled receptor expressed in the cardiovascular system. *Biochem Biophys Res Commun* **190**:1104-1109

Osada M, Yatomi Y, Ohmori T, Ikeda H, Ozaki Y (2002). Enhancement of sphingosine 1-phosphate-induced migration of vascular endothelial cells and smooth muscle cells by an EDG-5 antagonist. *Biochem Biophys Res Commun* **299**:483-487

Paik JH, Chae Ss , Lee MJ, Thangada S, Hla T (2001). Sphingosine 1-phosphate-induced endothelial cell migration requires the expression of EDG-1 and EDG-3 receptors and rho-dependent activation of alpha vbeta3- and beta1-containing integrins. *J Biol Chem* **276**:11830-11837

Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M, Miyano M (2000). Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* **289**:739-745

Panetti TS (2002). Differential effects of sphingosine 1-phosphate and lysophosphatidic acid on endothelial cells. *Biochim Biophys Acta* **1582**:190-196

Pappu R, Schwab SR, Cornelissen I, Pereira JP, Regard JB, Xu Y, Camerer E, Zheng YW, Huang Y, Cyster JG, Coughlin SR (2007). Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. *Science* **316**:295-298

Parent JL, Le Gouill C, Rola-Pleszczynski M, Stanková J (1996). Mutation of an aspartate at position 63 in the human platelet-activating factor receptor augments binding affinity but abolishes G-protein-coupling and inositol phosphate production. *Biochem Biophys Res Commun* **219**:968-975

Parrill AL, Wang D, Bautista DL, Van Brocklyn JR, Lorincz Z, Fischer DJ, Baker DL, Liliom K, Spiegel S, Tigyi G (2000). Identification of Edg1 receptor residues that recognize sphingosine 1-phosphate. *J Biol Chem* **275**:39379-39384

Perez DM, Karnik SS (2005). Multiple signaling states of G-protein-coupled receptors. *Pharmacol Rev* **57**:147-161

Pierce KL, Premont RT, Lefkowitz RJ (2002). Seven-transmembrane receptors. *Nat Rev Mol Cell Biol* **3**:639-650

Pronin AN, Benovic JL (1997). Regulation of the G protein-coupled receptor kinase GRK5 by protein kinase C. *J Biol Chem* **272**:3806-3812

Pyne S, Pyne N (2000). Sphingosine 1-phosphate signalling via the endothelial differentiation gene family of G-protein-coupled receptors. *Pharmacol Ther* **88**:115-131

Rasmussen SG, Jensen AD, Liapakis G, Ghanouni P, Javitch JA, Gether U (1999). Mutation of a highly conserved aspartic acid in the beta2 adrenergic receptor: constitutive activation, structural instability, and conformational rearrangement of transmembrane segment 6. *Mol Pharmacol* **56**:175-184

Rovati GE, Capra V, Neubig RR (2007). The highly conserved DRY motif of class A G protein-coupled receptors: beyond the ground state. *Mol Pharmacol* **71**:959-964

Roviezzo F, Bucci M, Delisle C, Brancaleone V, Di Lorenzo A, Mayo IP, Fiorucci S, Fontana A, Gratton JP, Cirino G (2006). Essential requirement for sphingosine kinase activity in eNOS-dependent NO release and vasorelaxation. *FASEB J* **20**:340-342

Rutherford C, Ord-Shrimpton FU, Sands WA, Pediani JD, Benovic JL, McGrath JC, Palmer TM (2005). Phosphorylation-independent internalisation and desensitisation of the human sphingosine-1-phosphate receptor S1P3. *Cell Signal* **17**:997-1009

Ryu Y, Takuwa N, Sugimoto N, Sakurada S, Usui S, Okamoto H, Matsui O, Takuwa Y (2002). Sphingosine-1-phosphate, a platelet-derived lysophospholipid mediator, negatively regulates cellular Rac activity and cell migration in vascular smooth muscle cells. *Circ Res* **90**:325–332

Salomone S, Yoshimura S, Reuter U, Foley M, Thomas SS, Moskowitz MA, Waeber C (2003). S1P3 receptors mediate the potent constriction of cerebral arteries by sphingosine-1-phosphate. *Eur J Pharmacol* **469**:125-134

Sanchez T, Hla T (2004). Structural and functional characteristics of S1P receptors. *J Cell Biochem* **92**:913-922

Sanchez T, Skoura A, Wu MT, Casserly B, Harrington EO, Hla T (2007). Induction of vascular permeability by the sphingosine-1-phosphate receptor-2 (S1P2R) and its downstream effectors ROCK and PTEN. *Arterioscler Thromb Vasc Biol* **27**:1312-1318

Sanna MG, Liao J, Jo E, Alfonso C, Ahn MY, Peterson MS, Webb B, Lefebvre S, Chun J, Gray N, Rosen H (2004). Sphingosine 1-phosphate (S1P) receptor subtypes S1P1 and S1P3, respectively, regulate lymphocyte recirculation and heart rate. *J Biol Chem* **279**:13839-13848

Sarnago S, Elorza A, Mayor F (1999). Agonist-dependent phosphorylation of the G protein-coupled receptor kinase 2 (GRK2) by Src tyrosine kinase. *J Biol Chem* **274**:34411-34416

Sato K, Kon J, Tomura H, Osada M, Murata N, Kuwabara A, Watanabe T, Ohta H, Ui M, Okajima F (1999). Activation of phospholipase C-Ca2+ system by sphingosine 1-phosphate in CHO cells transfected with Edg-3, a putative lipid receptor. *FEBS Lett* **443**:25-30

Scheer A, Costa T, Fanelli F, De Benedetti PG, Mhaouty-Kodja S, Abuin L, Nenniger-Tosato M, Cotecchia S (2000). Mutational analysis of the highly conserved arginine within the Glu/Asp-Arg-Tyr motif of the alpha(1b)-adrenergic receptor: effects on receptor isomerization and activation. *Mol Pharmacol* **57**:219-231

Scherer EQ, Lidington D, Oestreicher E, Arnold W, Pohl U, Bolz SS (2006). Sphingosine-1-phosphate modulates spiral modiolar artery tone: a potential role in vascular-based inner ear pathologies? *Cardiovasc Res* **70**:79–87

Schmouder R, Serra D, Wang Y, Kovarik JM, DiMarco J, Hunt TL, Bastien MC (2006). FTY720: placebo-controlled study of the effect on cardiac rate and rhythm in healthy subjects. *J Clin Pharmacol* **46**:895-904

Siess W (2002). Athero- and thrombogenic actions of lysophosphatidic acid and sphingosine-1-phosphate. *Biochim Biophys Acta* **1582**:204-215

Singleton PA, Dudek SM, Chiang ET, Garcia JG (2005). Regulation of sphingosine 1-phosphate-induced endothelial cytoskeletal rearrangement and barrier enhancement by S1P1 receptor, PI3 kinase, Tiam1/Rac1, and alpha-actinin. *FASEB J* **9**:1646-1656

Singleton PA, Dudek SM, Ma SF, Garcia JG (2006). Transactivation of sphingosine 1-phosphate receptors is essential for vascular barrier regulation. Novel role for hyaluronan and CD44 receptor family. *J Biol Chem* **281**:34381-34393

Smith RD, Baukal AJ, Zolyomi A, Gaborik Z, Hunyady L, Sun L, Zhang M, Chen HC, Catt KJ (1998). Agonist-induced phosphorylation of the endogenous AT1 angiotensin receptor in bovine adrenal glomerulosa cells. *Mol Endocrinol* **12**:634-644

Spiegel S, Milstien S (2003). Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat Rev Mol Cell Biol* **4**:397-407

Stout BD, Clarke WP, Berg KA (2002). Rapid desensitization of the serotonin(2C) receptor system: effector pathway and agonist dependence. *J Pharmacol Exp Ther* **302**:957-962

Sugiyama A, Yatomi Y, Ozaki Y, Hashimoto K (2000). Sphingosine 1-phosphate induces sinus tachycardia and coronary vasoconstriction in the canine heart. *Cardiovasc Res* **46**:119-125

Suzuki S, Enosawa S, Kakefuda T, Shinomiya T, Amari M, Naoe S, Hoshino Y, Chiba K (1996). A novel immunosuppressant, FTY720, with a unique mechanism of action, induces long-term graft acceptance in rat and dog allotransplantation. *Transplantation* **61**:200-205

Tamama K, Okajima F (2002). Sphingosine 1-phosphate signaling in atherosclerosis and vascular biology. *Curr Opin Lipidol* **13**:489-495

Tamama K, Kon J, Sato K, Tomura H, Kuwabara A, Kimura T, Kanda T, Ohta H, Ui M, Kobayashi I, Okajima F (2001). Extracellular mechanism through the Edg family of receptors might be responsible for sphingosine-1-phosphateinduced regulation of DNA synthesis and migration of rat aortic smooth-muscle cells. *Biochem J* **353**:139–146.

Tani M, Ito M, Igarashi Y (2007). Ceramide/sphingosine/sphingosine 1-phosphate metabolism on the cell surface and in the extracellular space. *Cell Signal* **19**:229-237

Terai K, Soga T, Takahashi M, Kamohara M, Ohno K, Yatsugi S, Okada M, Yamaguchi T (2003). Edg-8 receptors are preferentially expressed in oligodendrocyte lineage cells of the rat CNS. *Neuroscience* **116**:1053-1062

Thudichum JLW (1884). A treatise on the chemical constitution of the brain. London: Bailliere, Tindall&Co.

Tölle M, Levkau B, Keul P, Brinkmann V, Giebing G, Schönfelder G, Schäfers M, von Wnuck Lipinski K, Jankowski J, Jankowski V, Chun J, Zidek W, Van der Giet M (2005). Immunomodulator FTY720 Induces eNOS-dependent arterial vasodilatation via the lysophospholipid receptor S1P3. *Circ Res* **96**:913-920

Tosaka M, Okajima F, Hashiba Y, Saito N, Nagano T, Watanabe T, Kimura T, Sasaki T (2001). Sphingosine 1-phosphate contracts canine basilar arteries in vitro and in vivo: possible role in pathogenesis of cerebral vasospasm. *Stroke* **32**:2913-2919

Uhlenbrock K, Gassenhuber H, Kostenis E (2002). Sphingosine 1-phosphate is a ligand of the human gpr3, gpr6 and gpr12 family of constitutively active G protein-coupled receptors. *Cell Signal* **14**:941-953

Urban JD, Clarke WP, von Zastrow M, Nichols DE, Kobilka B, Weinstein H, Javitch JA, Roth BL, Christopoulos A, Sexton PM, Miller KJ, Spedding M, Mailman RB (2007). Functional selectivity and classical concepts of quantitative pharmacology. *J Pharmacol Exp Ther* **320**:1-13

Van Brocklyn JR, Lee MJ, Menzeleev R, Olivera A, Edsall L, Cuvillier O, Thomas DM, Coopman PJ, Thangada S, Liu CH, Hla T, Spiegel S (1998). Dual actions of sphingosine-1-phosphate: extracellular through the Gi-coupled receptor Edg-1 and intracellular to regulate proliferation and survival. *J Cell Biol* **142**:229-240

Van Brocklyn JR, Gräler MH, Bernhardt G, Hobson JP, Lipp M, Spiegel S (2000). Sphingosine-1-phosphate is a ligand for the G protein-coupled receptor EDG-6. *Blood* **95**:2624-2629

Vaidehi N, Floriano WB, Trabanino R, Hall SE, Freddolino P, Choi EJ, Zamanakos G, Goddard WA (2002). Prediction of structure and function of G protein-coupled receptors. *Proc Natl Acad Sci U S A* **99**:12622-12627

Visentin B, Vekich JA, Sibbald BJ, Cavalli AL, Moreno KM, Matteo RG, Garland WA, Lu Y, Yu S, Hall HS, Kundra V, Mills GB, Sabbadini RA (2006). Validation of an anti-sphingosine-1-phosphate antibody as a potential therapeutic in reducing growth, invasion, and angiogenesis in multiple tumor lineages. *Cancer Cell* **9**:225-238

Waeber C, Blondeau N, Salomone S. Waeber C, Blondeau N, Salomone S (2004). Vascular sphingosine-1-phosphate S1P1 and S1P3 receptors. *Drug News Perspect* **17**:365-382

Wang F, Van Brocklyn JR, Hobson JP, Movafagh S, Zukowska-Grojec Z, Milstien S, Spiegel S (1999). Sphingosine 1-phosphate stimulates cell migration through a Gi-coupled cell surface receptor. Potential involvement in angiogenesis. *J Biol Chem* **274**:35343–35350

Waters CM, Long J, Gorshkova I, Fujiwara Y, Connell M, Belmonte KE, Tigyi G, Natarajan V, Pyne S, Pyne NJ (2006). Cell migration activated by platelet-derived growth factor receptor is blocked by an inverse agonist of the sphingosine 1-phosphate receptor-1. *FASEB J* **20**:509-511

Willars GB (2006). Mammalian RGS proteins: multifunctional regulators of cellular signalling. *Semin Cell Dev Biol* **17**:363-376

Windh RT, Lee MJ, Hla T, An S, Barr AJ, Manning DR (1999). Differential coupling of the sphingosine 1-phosphate receptors Edg-1, Edg-3, and H218/Edg-5 to the G(i), G(q), and G(12) families of heterotrimeric G proteins. *J Biol Chem* **274**:27351-27358

Wong WS, Rosoff PM (1996). Pharmacology of pertussis toxin B-oligomer. *Can J Physiol Pharmacol* **74**:559-564

Woodside DG (2002). Dancing with multiple partners. *Sci STKE* **2002**:PE14

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

Yamaguchi F, Tokuda M, Hatase O, Brenner S (1996). Molecular cloning of the novel human G protein-coupled receptor (GPCR) gene mapped on chromosome 9. *Biochem Biophys Res Commun* **227**:608-614

Yamaguchi H, Kitayama J, Takuwa N, Arikawa K, Inoki I, Takehara K, Nagawa H, Takuwa Y (2003). Sphingosine-1-phosphate receptor subtype-specific positive and negative regulation of Rac and haematogenous metastasis of melanoma cells. *Biochem J* **374**:715–722.

Yamazaki Y, Kon J, Sato K, Tomura H, Sato M, Yoneya T, Okazaki H, Okajima F, Ohta H (2000). Edg-6 as a putative sphingosine 1-phosphate receptor coupling to Ca(2+) signaling pathway. *Biochem Biophys Res Commun* **268**:583-289

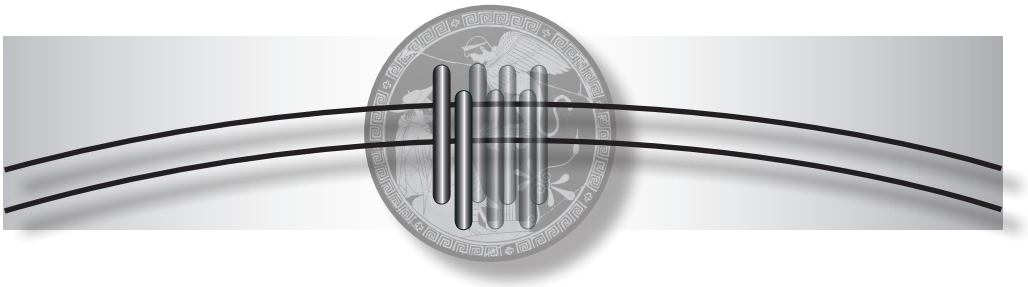
Yang AH, Ishii I, Chun J (2002). In vivo roles of lysophospholipid receptors revealed by gene targeting studies in mice. *Biochim Biophys Acta* **1582**:197-203

Yatomi Y, Ohmori T, Rile G, Kazama F, Okamoto H, Sano T, Satoh K, Kume S, Tigyi G, Igarashi Y, Ozaki Y (2000). Sphingosine 1-phosphate as a major bioactive lysophospholipid that is released from platelets and interacts with endothelial cells. *Blood* **96**:3431-3438

Yatomi Y, Ozaki Y, Ohmori T, Igarashi Y (2001). Sphingosine1-phosphate: synthesis and release. *Prostaglandins Other Lipid Mediat* **64**:107–122

Young N, Van Brocklyn JR (2006). Signal transduction of sphingosine-1-phosphate G protein-coupled receptors. *ScientificWorldJournal* **6**:946-966

Zondag GC, Postma FR, Etten IV, Verlaan I, Moolenaar WH (1998). Sphingosine 1-phosphate signalling through the G-protein-coupled receptor Edg-1. *Biochem J* **330**:605-609



BML-241,
a selective $S1P_3$ receptor antagonist?

Chapter 2

BML-241, a selective $S1P_3$ receptor antagonist?

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Abstract

Based upon a screening of $[Ca^{2+}]_i$ responses in HeLa cells overexpressing S1P receptors, it was suggested that BML-241 is a lead compound to develop a selective $S1P_3$ receptor antagonist. We have studied the proposed selective antagonistic properties of BML-241 for the $S1P_3$ receptor in more detail.

Chinese hamster ovary (CHO) cells stably transfected with the $S1P_3$, $S1P_2$ or α_{1A}-adrenoceptor were used to investigate the effect of BML-241 on $[Ca^{2+}]_i$ elevations mediated via different receptors. CHO-K1 cells were used to study ATP-induced $[Ca^{2+}]$ elevations. Also the effect on $S1P_3$-mediated inhibition of forskolin-induced cAMP accumulation and on binding to the α_{1A}-adrenoceptor were investigated. In addition, the effect of BML-241 on the inhibition of phenylephrine-induced contraction of rat mesenteric artery was studied in an organ bath.

High concentrations of BML-241 (10 μM) inhibited $[Ca^{2+}]_i$ elevations induced by $S1P_3$ and $S1P_2$ receptor stimulation, whereas lower concentrations were ineffective. The high concentration of BML-241 also inhibited $[Ca^{2+}]_i$ increases via purinergic and α_{1A}-adrenoceptor stimulation. Moreover, BML-241 (10 μM) inhibited α_{1A}-adrenoceptor-mediated contraction of rat mesenteric artery. However, it did not displace [^{3}H]-prazosin from the α_{1A}-adrenoceptors in concentrations up to 100 μM. BML-241 (10 μM) did not affect the $S1P_3$-mediated decrease of forskolin-induced cAMP accumulation. We conclude that BML-241 is a low potency inhibitor of $[Ca^{2+}]_i$ responses, rather than a specific antagonist of the $S1P_3$ receptor.

Introduction

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid found in high concentrations in blood (Yatomi et al.,1997), it is produced and stored in platelets, from which it is released upon stimulation. S1P can also be produced by many other cell types from membrane phospholipids to act in an auto- and/or paracrine manner.

S1P regulates many different biological functions like cell growth, proliferation, apoptosis, lymphocyte recirculation and angiogenesis via specific receptors. The first S1P receptor was discovered as an abundantly expressed gene in differentiating endothelial cells (Hla et al.,1990). Though orphan at first, the product of the endothelial differentiation gene 1 (EDG-1) turned out to have high affinity for S1P and was, therefore, renamed $S1P_1$ (Lee et al., 1998). The family of S1P receptors currently consists of 5 members with close homology. The $S1P_1$, $S1P_2$ and $S1P_3$ receptors are ubiquitously expressed, whereas the $S1P_4$ and $S1P_5$ receptor show a more restricted expression pattern (Alewijnse et al., 2004). The $S1P_1$ receptor predominantly activates G_i-proteins, the $S1P_2$ and $S1P_3$ receptor activate G_i, $G_{q/11}$ and $G_{12/13}$-proteins and the $S1P_4$ and $S1P_5$ receptors activate G_i and $G_{12/13}$-proteins (Spiegel et al., 2003).

The specific role of S1P receptor subtypes has predominantly been studied using genetic approaches because of the limited availability of selective agonists and antagonists. Research with knockout mice has for instance shown a crucial role for the $S1P_1$ receptor in the formation of blood vessels (Liu et al., 2000). However, pharmacological tools are still needed.

In 2002, a new compound, BML-241, was reported from a screening of $[Ca^{2+}]$ responses in HeLa cells overexpressing S1P receptors and was proposed to be a lead compound to develop a selective antagonist of the $S1P_3$ receptor (Koide et al., 2002). However, conclusions were based on measurements of one BML-241 concentration and on the comparison of only the $S1P_1$ and $S1P_3$ receptor in one assay. Therefore, we have investigated the proposed selectivity of the $S1P_3$ receptor antagonistic properties of BML-241 in more detail.

Materials and Methods

Materials

pcDNA3.1 containing the entire coding region of the human $S1P_2$ or $S1P_3$ receptor was purchased from UMR cDNA Resource Center (Rolla, MO, USA). Cell culture media, hygromycine B, Lipofectamine™ 2000, Optimem, Hank's Balanced Salt Solution (HBSS), pOG44, pcDNA3.1/HisA/C, pcDNA5/FRT/TO and CHO-FlpIn cells, were obtained from Invitrogen (Breda, The Netherlands). Pluronic acid and Fluo-4 AM were obtained from Molecular Probes (via Invitrogen). Foetal calf serum (FCS), enzyme free cell dissociation buffer and penicillin/streptomycin were obtained from Gibco (via Invitrogen). LANCE™ cAMP 384 kit, white 384 well optiplates and Microscint-O were obtained from Perkin Elmer (Zaventem, Belgium). Restriction enzymes (BamHI, ApaI, XhoI and HindIII) were obtained from Fermentas Life Sciences (St. Leon-Rot, Germany). S1Pwas obtained from Avanti-Polar Lipids, Inc (via Instruchemie B.V., Delfzijl, The Netherlands). Probenecid, Hepes, EGTA, Triton X-100, 3-isobutyl-1-methylxanthine, bovine serum albumin (BSA) (fatty acid free) and activated charcoal were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). Clear bottom black 96 well plates were obtained from Greiner Bio One (Alphen aan den Rijn, The Netherlands). BML-241 (2(R,S)-undecylthiazolidine-4(R)-carboxylic acid) was obtained from Biomol (via Tebu-Bio, Heerhugowaard, The Netherlands). CHO-K1 cells were purchased from ECACC (Zwijndrecht, The Netherlands). [^{3}H]-prazosin (specific activity 80 Ci/mmol) was obtained from Amersham (Diegem, Belgium). CHO cells stably expressing the human α_{1A}-adrenoceptor at a density of approximately 2 pmol/mg protein were generated as previously reported (Keffel et al., 2000).

Molecular cloning

An N-terminal HisG-tag was added to the $S1P_2$ or $S1P_3$ receptor via cloning into pcDNA3.1/HisC or pcDNA3.1/HisA using BamHI & ApaI or BamHI & XhoI digestion, respectively. A second cloning step was done using HindIII & ApaI or HindIII & XhoI to clone the HisG-tagged $S1P_2$ or $S1P_3$ receptor into the expression vector pcDNA5/FRT/TO using standard cloning techniques.

Transfection & cell culture

Transfection of plasmids into CHO-FlpIn cells was done according to the manufacturer's protocol with Lipofectamine™ 2000. 600 µg/ml hygromycin B was used to select for positive clones, which were subsequently separately grown to confluency. Expression of the $S1P_2$ or $S1P_3$ receptor was confirmed by immunofluorescence against the HisG-tag. CHO-FlpIn cells stably expressing the $S1P_2$ or $S1P_3$ receptor were passaged 1:5 every two or three days in F-12 Nutrient Mixture (Ham) with L-glutamine, supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 313 µg/ml hygromycin B and 10 % charcoal-stripped FCS. CHO-K1 cells were passaged 1:10 every two or three days in F-12 Nutrient Mixture (Ham) with L-glutamine, supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10 % FCS. CHO-K1 cells stably expressing α_{1A}-adrenoceptor were cultured as described previously (Keffel et al., 2000).All cell lines were cultured at 37 °C in humidified air containing 5 % CO_2.

Intracellular calcium measurement

Cells were plated in a black, clear bottom 96 wells plate at 40,000 cells/well. After growth overnight, cells were loaded for 1 hour with basic buffer (HBSS containing 20 mM Hepes and 2.5 mM probenecid) containing 4 µM Fluo-4 AM ester and 0.42 % v/v pluronic acid and incubated at 37 °C. After loading, cells were washed twice with basic buffer and incubated at 37 °C with basic buffer in the presence or absence of BML-241 for 60 min. Fluorescence was measured at 37 °C using an excitation filter at 485 nm and emission filter at 520 nm on a NOVOstar (BMG Labtech, via Isogen, IJsselstein, The Netherlands). After measuring the basal level for 10 s, the ligand was added at 10 % v/v basic buffer. After 50 sec 10 % v/v Triton X-100 in basic buffer was added at 10 % v/v to determine the maximal signal (F_{max}). After 20 sec 0.1 M EGTA in basic buffer was added at 10 % v/v to determine the minimal signal (F_{min}). The increase in $[Ca^{2+}]_i$ upon ligand stimulation was calculated as the difference between the $[Ca^{2+}]_i$ for the basal level and after adding a ligand. The $[Ca^{2+}]_i$ was calculated via the equation: $[Ca^{2+}]_i = Kd * ((F-F_{min})/(F_{max}-F))$. Kd is the dissociation constant of the binding of Fluo-4 to calcium (345 nM).

cAMP assay

The LANCE™ cAMP 384 kit was used to determine cAMP concentrations according to the manufacturer's protocol. CHO-FlpIn cells stably expressing the $S1P_3$ receptor were incubated for 1 hour with or without 10 µM BML-241 in F-12 Nutrient Mixture (Ham) with L-glutamine and detached from the surface using enzyme free cell dissociation buffer. Cells were washed once with HBSS and subsequently resuspended in stimulation buffer, containing HBSS with 0.5 mM 3-isobutyl-1-methylxanthine, 0.05% BSA and 5 mM Hepes.

Stimulation mixtures consisted of stimulation buffer with 3 µM forskolin, 10 µM BML-241 or vehicle and the concentration response curve of S1P ranging from 10 pM to 1 µM.

Cells were added to the stimulation mixtures 1:1 in a 384 well optiplate at 2500 cells/well and stimulated for 60 minutes. Detection of the cAMP formed during stimulation was done according to the manufacturer's protocol. Measurements were done on a platereader (Victor 2, Wallac, Perkin Elmer) 3 hours after adding detection buffer and antibody mixture.

Radioligand binding studies

Confluent 20 cm dishes with CHO-FlpIn cells stably expressing α_{1A}-adrenoceptor (Keffel et al., 2000) were washed once in PBS. Cells were scraped in ice-cold PBS, and spun down for 10 min at 200xg, 4 °C. The pellet was washed once in 50 mM Tris, 0.5 mM EDTA and then homogenised in 50 mM Tris, 0.5 mM EDTA on ice using a Turrax. The suspension was spun down for 20 min at 50,000xg at 4 °C. The pellet was resuspended in 50 mM Tris, 0.5 mM EDTA and homogenised again. Protein concentration was determined according to Bradford (Bio-Rad, Veenendaal, The Netherlands). Competition binding studies were performed as previously described (Keffel et al., 2000). Briefly, incubations were performed for 45 min at 25 °C in a total volume of 1 ml containing [^{3}H]-prazosin and 50 mM Tris, 0.5 mM EDTA, pH 7.5. Non-specific binding was defined in the presence of 10 µM phentolamine. The incubations were terminated by rapid vacuum filtration over Whatman GF/C filters, which were washed with ice-cold binding buffer. After addition of Microscint-O, radioactivity of bound fraction on the filter was counted by a TopCount scintillation counter (Perkin Elmer, Zaventem, Belgium).

Vascular contraction studies

The experiments followed a protocol approved by the institutional Animal Ethical Committee. Adult male Wistar rats (280 – 320 g, Charles Rivers, Maastricht, The Netherlands) were anaesthetized by i.p. injection of 75 mg/kg pentobarbitone (O.B.G., Utrecht, The Netherlands). 500 I.U. heparin (Leo Pharma B.V., Weesp, The Netherlands) was administered i.p. to prevent blood coagulation. The mesenteric vascular bed was exteriorized 10 cm below the pylorus and immediately placed in Krebs-Henseleit buffer (118.0 mM NaCl, 4.7 mM KCl, 25.0 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.1 mM KH_2PO_4 and 5.6 mM glucose) at room temperature, aerated with 5 % CO_2 / 95 % O_2, pH 7.4. Mesenteric vessels from the third order branches, with a length of about 3 mm, were isolated from fat and surrounding tissue, and a 40 µm diameter stainless-steel wire was inserted into the lumen of the vessel. The vessel was dissected and the segments were then transferred into the organ baths of a 4-channel wire myograph (Danish Myo Technology, Aarhus, Denmark) and subjected to a normalization procedure according to Mulvany & Halpern (Mulvany & Halpern, 1977). In short, the individual circumference was adjusted to 90 % of the value that the particular vessel would have had at a transmural pressure of 100 mmHg. Afterwards, the arteries were equilibrated for an additional 45 min and the buffer was refreshed every 20 min. The preparations were contracted twice for 10 min with a depolarizing high K^+ Krebs-Henseleit solution (100 mM NaCl was replaced by 120 mM KCl) at intervals of 15 min. Subsequently, the vessels were precontracted with the α_1-adrenoceptor agonist phenylephrine (0.3 µM). After reaching a steady level, one concentration of the endothelium-dependent vasodilator methacholine (10 µM) was added to assess the endothelial integrity. Vessels were excluded when relaxation was less than 80 %. After washing, again 100 mM KCl was added to the vessel segments to obtain the maximal contractile response. After washing and an additional 20 min equilibration, 10 µM BML-241 was added to the bathing solution. After 30 min, a cumulative concentration response curve (30 nM – 30 µM) for phenylephrine was constructed. Isometric force of contraction was measured continuously and all data presented are normalized to the contractile response obtained by the third 100 mM KCl-induced contraction.

Data analysis

Concentration response curves were analysed by fitting sigmodial functions to the experimental data using Prism 4 (Graphpad Software, Inc., San Diego, CA, USA). Data are expressed as means ± s.e.m. Differences between two groups were compared using a paired Student's t-test. One-way ANOVA with a Dunnett's correction was used where appropriate. $p<0.05$ was considered significant.

Results

Effect of BML-241 on S1P-induced $[Ca^{2+}]_i$ elevation in CHO cells stably expressing the $S1P_3$ receptor

S1P concentration-dependently increased the $[Ca^{2+}]_i$ in CHO-FlpIn cells stably expressing the $S1P_3$ receptor. The calcium tracing shows a rapid ligand-induced increase followed by a decrease (Figure 1A), the former being characterized by a pEC_{50} of 8.1 ± 0.2 and an E_{max} of 2837 ± 419 nM (n = 9, Figure 2A). A 60 min pre-incubation with 10 µM BML-241 caused a rightward shift of the concentration-response curve without affecting the maximum responses (pEC_{50} = 7.2 ± 0.2, E_{max} = 2697 ± 403 nM, n = 8, $p<0.05$, Figure 2A). Lower concentrations of BML-241 (1 or 3 µM) did not significantly influence the $[Ca^{2+}]_i$ response (data not shown).

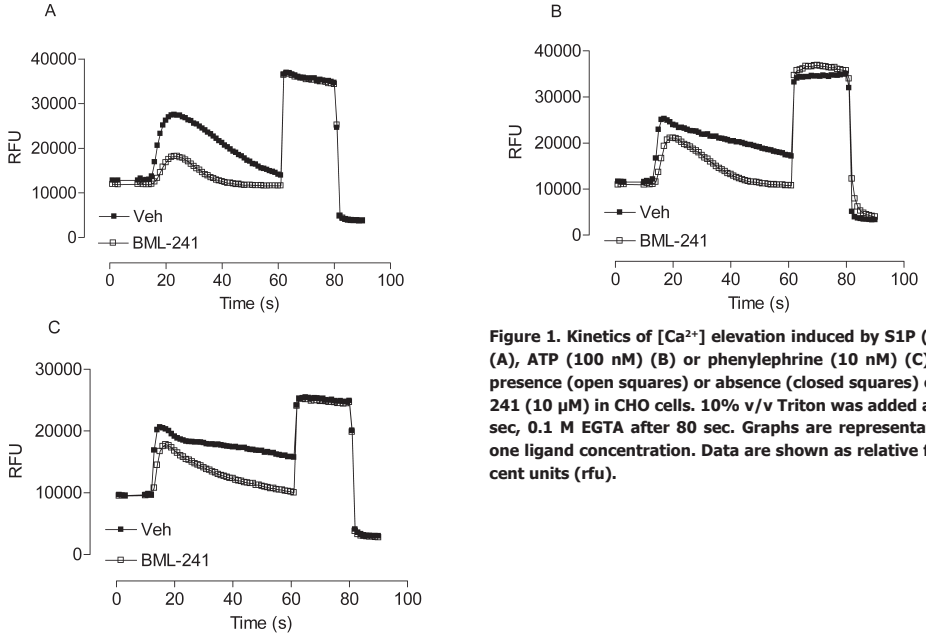

Figure 1. Kinetics of $[Ca^{2+}]$ elevation induced by S1P (10 nM) (A), ATP (100 nM) (B) or phenylephrine (10 nM) (C) in the presence (open squares) or absence (closed squares) of BML-241 (10 µM) in CHO cells. 10% v/v Triton was added after 60 sec, 0.1 M EGTA after 80 sec. Graphs are representative for one ligand concentration. Data are shown as relative fluorescent units (rfu).

Effect of BML-241 on S1P-induced $[Ca^{2+}]_i$ elevation in CHO cells stably expressing the $S1P_2$ receptor

S1P also concentration-dependently increased the $[Ca^{2+}]_i$ in CHO-FlpIn cells stably expressing the $S1P_2$ receptor. The concentration response curve was significantly shifted to the right after a pre-incubation with 10 µM BML-241, whereas the maximal response was not affected (pEC_{50} = 7.7 ± 0.1 vs. 7.3 ± 0.1, $p<0.05$, E_{max} = 2698 ± 257 vs 2309 ± 359 nM, n = 6-7, Figure 2B). However, lower concentrations of BML-241 (1 or 3 µM) did not show such inhibition (data not sown).

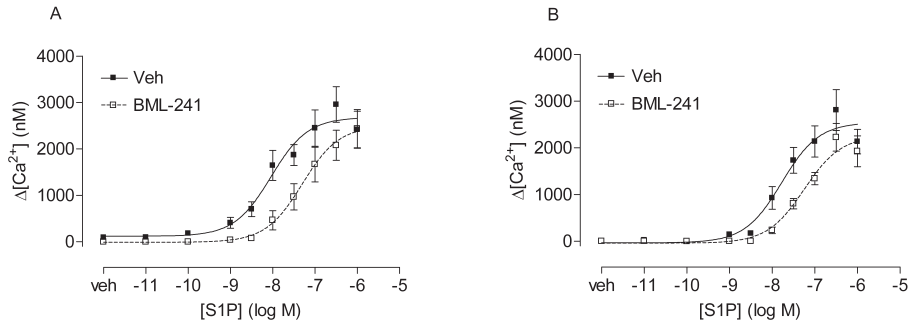

Figure 2. Concentration response curve of S1P-induced $[Ca^{2+}]$ elevation in CHO-FlpIn cells stably expressing the $S1P_3$-receptor (n=9) (A) or the $S1P_2$-receptor (B) in the presence (open squares) or absence (closed squares) of BML-241 (10 µM) (n=6-7).

Effect of BML-241 on $[Ca^{2+}]_i$ elevation in CHO cells via the purinergic receptors and α_{1A}-adrenoceptor

In CHO-cells expressing the purinergic receptor or α_{1A}-adrenoceptor ATP and phenylephrine also increased $[Ca^{2+}]_i$. However, the fluorescent signal remained elevated longer compared to S1P-mediated stimulation (Figure 1A,B,C). Therefore, we measured the effect of BML-241 on peak responses ('early') as well as after 60 s ('late'). Pre-incubation with BML-241 (10 µM) caused a significant rightward shift of the ATP-induced $[Ca^{2+}]$ elevation in the early phase (pEC_{50} = 6.9 ± 0.1 vs. 6.4 ± 0.1, n=5, $p<0.05$, Figure 3A), but had no significant effect on the late response (pEC_{50} = 6.3 ± 0.1 vs. 6.1 ± 0.1, n=5). On the other hand, this concentration of BML-241 did not significantly alter the E_{max} of the early response (E_{max} = 1463 ± 72 vs. 1536 ± 152 nM, n=5), but significantly decreased the E_{max} of the late response (E_{max} = 972 ± 43 vs. 697 ± 62 nM, n=5, $p<0.05$). Similarly, BML-241 (10 µM) significantly shifted the phenylephrine-induced $[Ca^{2+}]_i$ elevation to the right for the early

phase (pEC_{50} = 8.4 ± 0.1 vs. 8.1 ± 0.1, n=5, p<0.05, Figure 3B), as well as for the late phase (pEC_{50} = 7.9 ± 0.1 vs. 7.7 ± 0.1, n=5, p<0.05). BML-241 (10 µM) did not significantly alter the E_{max} of the early response (E_{max} = 1719 ± 98 vs. 1693 ± 148 nM, n=5), but significantly decreased the late response (E_{max} = 1241 ± 58 vs. 889 ± 43 nM, n=5, p<0.05).

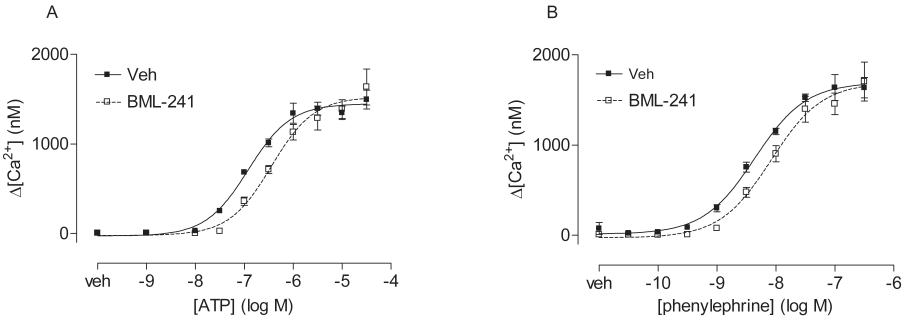

Figure 3. Concentration response curve of the early phase of ATP-induced (A) and phenylephrine-induced (B) [Ca^{2+}] elevation in CHO-K1 cells in the presence (open squares) or absence (closed squares) of BML-241 (10 µM) (n=5).

Effect of BML-241 on phenylephrine-induced contraction of the mesenteric artery

To investigate non-selective effects of BML-241 on a tissue level, an organ bath setup was used. In mesenteric artery preparations phenylephrine causes contraction via the α_1-adrenoceptor. Addition of BML-241 (10 µM) caused a significant rightward shift of the concentration response curve for phenylephrine (pEC_{50} = 5.9 ± 0.1 vs. 5.4 ± 0.1, n=6-7, p<0.05, Figure 4) and significantly decreased the E_{max} (E_{max} = 160 ± 6 vs. 132 ± 9 %, n=6-7, p<0.05, Figure 4). BML-241 had no significant influence on KCl-induced constriction (data not shown).

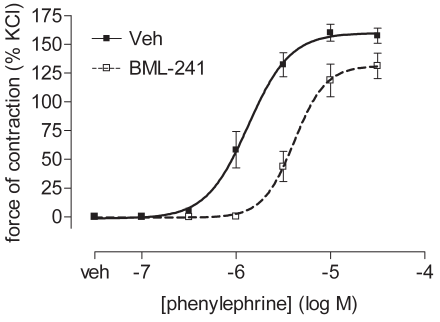

Figure 4. Phenylephrine-induced contraction of rat mesenteric artery in the presence (open squares) or absence (closed squares) of BML-241 (10 µM) (n=6-7). Data are expressed as percentage of the third KCl (120 mM)-induced contraction.

Effect of BML-241 on binding of [^{3}H]-prazosin to the α_{1A}-adrenoceptor

In competition binding experiments, BML-241 in concentrations up to 100 µM did not cause detectable inhibition of radioligand binding to α_{1A}-adrenoceptors, whereas phenylephrine as the positive control exhibited a concentration-dependent displacement of the radioligand (Figure 5).

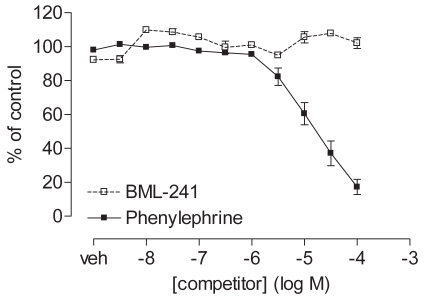

Figure 5. Displacement of [^{3}H]-prazosin on membranes of CHO cells stably expressing the human α_{1A}-adrenoceptor by increasing concentrations of phenylephrine or BML-241 (n=3). Data are expressed as % of control.

Effect of BML-241 on $S1P_3$-induced inhibition of cAMP accumulation

In CHO-FlpIn cells stably expressing the $S1P_3$ receptor, S1P inhibited forskolin-induced cAMP accumulation in a concentration-dependent manner (pEC_{50} = 7.8 ± 0.4, n=4). Pre-incubation with BML-241 (10µM) did not have a significant influence on the S1P-induced inhibition of cAMP accumulation (pEC_{50} = 8.1 ± 0.5, n=4,). In mock transfected CHO cells, S1P did not inhibit forskolin-induced cAMP accumulation (Figure 6).

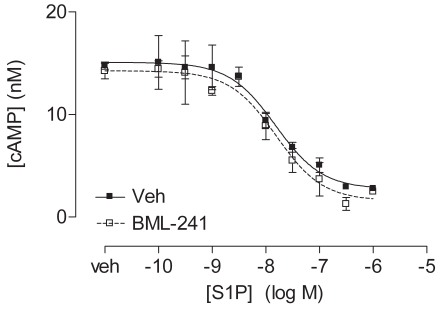

Figure 6. Concentration response curve of S1P-induced inhibition of forskolin-induced cAMP accumulation in the presence (open squares) or absence (closed squares) of BML-241 (10 µM) in CHO-FlpIn cells stably expressing the $S1P_3$-receptor (n=4).

Discussion

Research on S1P receptors has been hampered by a lack of specific ligands, particularly reagents which allow investigating the role of endogenous S1P. The $S1P_3$ receptor can stimulate phospholipase C, activate rho and regulate ERK1/2 activation (Watterson et al., 2005). Specific and particularly subtype-selective antagonists could help considerably to better understand the physiology of S1P responses as well as the mechanisms of drugs targeted at this system; moreover such antagonists have the potential to become useful drugs by themselves.

In 2002, it was reported from a screening of HeLa cells that a single high concentration of BML-241 (10 µM) inhibited the $S1P_3$-induced $[Ca^{2+}]_i$ response by 37 %, whereas $S1P_1$-induced responses were not affected (Koide et al., 2002). Based upon these data, BML-241 was proposed to be a lead compound to develop a selective antagonist of the $S1P_3$ receptor. Our study confirms that a high concentration of BML-241 indeed inhibits the S1P-induced $[Ca^{2+}]_i$ elevation in cells overexpressing the $S1P_3$ receptor. However, this high concentration (10 µM), was not selective for the $S1P_3$ receptor, but also affected the $S1P_2$-mediated $[Ca^{2+}]_i$ elevation.

To determine the specificity of BML-241 for S1P receptors, effects on purinergic receptors and α_1-adrenoceptors were studied using ATP and phenylephrine, respectively. Interestingly, a difference in kinetics of the $[Ca^{2+}]_i$ signal was observed compared to S1P stimulated cells stably expressing the $S1P_2$ or $S1P_3$ receptor. The purinergic receptor agonist ATP and the α_{1A}-adrenoceptor agonist phenylephrine also rapidly increased $[Ca^{2+}]_i$, but the fluorescent signal remained elevated longer than after S1P receptor stimulation. Therefore, the effect of BML-241 was investigated in the 'early' and 'late' phases separately. BML-241 (10 µM) caused a significant rightward shift of the EC_{50} in the early phase, but not in the late phase for the ATP-induced $[Ca^{2+}]_i$ response. However, for the phenylephrine-induced $[Ca^{2+}]_i$ response, BML-241 caused a significant rightward shift of the EC_{50} of the early and the late phase. The maximal response for both the ATP-induced and the phenylephrine-induced $[Ca^{2+}]_i$ elevation was significantly decreased by BML-241 (10 µM) in the late phase but not in the early phase.

The relevance of the lack of selectivity for S1P receptors was confirmed at the tissue level by the inhibitory effect of BML-241 on the phenylephrine-induced contraction in a rat mesenteric artery preparation. The combination of BML-241 being

a low-potency and a non-selective antagonist of $[Ca^{2+}]_i$ responses could be due to low affinity binding to multiple receptors. However, BML-241 did not displace [^{3}H]-prazosin from the α_{1A}-adrenoceptor, even at very high concentrations (100 μM), indicating that BML-241 did not act at the receptor level. Based upon these results, we conclude that BML-241 is a low-potency functional antagonist of $[Ca^{2+}]_i$ elevation rather than a specific $S1P_3$ receptor antagonist. In line with this conclusion, BML-241 showed a lack of antagonism on the G_i-mediated inhibitory effect of the $S1P_3$ receptor on forskolin-induced cAMP production. Recently, expression of $S1P_1$, $S1P_2$ and $S1P_4$ receptor mRNA was shown in CHO-K1 cells (Holdsworth et al., 2005). Hence, the S1P-induced inhibition of forskolin-induced cAMP accumulation could be caused via these endogenous receptors instead of the stably transfected $S1P_3$ receptor. However, in our mock transfected CHO-FlpIn cells, there is no effect of S1P on the forskolin-induced cAMP accumulation. Therefore, the effect of S1P can only be caused via the stably transfected $S1P_3$ receptor and not by endogenous S1P receptors. Due to the complexity of calcium signaling, identification of the low-affinity molecular target of BML-241 remains unclear and is beyond the scope of this paper. However, these results show that possible new S1P receptor antagonists, like BML-241, but also the new compound VPC23019 (Davis et al., 2005), a $S1P_1$/$S1P_3$ antagonist, for example, should be investigated thoroughly.

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References

Alewijnse AE, Peters SL, Michel MC (2004). Cardiovascular effects of sphingosine-1-phosphate and other sphingomyelin metabolites. *Br J Pharmacol* **143**:666-684

Davis MD, Clemens JJ, Macdonald TL, Lynch KR (2005). Sphingosine 1-phosphate analogs as receptor antagonists. *J Biol Chem* **280**:9833-9841

Hla T, Maciag T (1990). An abundant transcript induced in differentiating human endothelial cells encodes a polypeptide with structural similarities to G-protein-coupled receptors. *J Biol Chem* **265**:9308-9313

Holdsworth G, Slocombe P, Hutchinson G, Milligan G (2005).Analysis of endogenous S1P and LPA receptor expression in CHO-K1 cells. *Gene* **350**:59-63

Keffel S, Alexandrov A, Goepel M, Michel MC (2000). Alpha(1)-adrenoceptor subtypes differentially couple to growth promotion and inhibition in Chinese hamster ovary cells. *Biochem Biophys Res Commun* **272**:906-911

Koide Y, Hasegawa T, Takahashi A, Ende A, Mochizuki N, Nakagawa M, Nishida A (2002). Development of novel EDG3 antagonists using a 3D database search and their structure-activity relationships. *J Med Chem* **45**:4629-4638

Lee MJ, Van Brocklyn JR, Thangada S, Liu CH, Hand AR, Menzeleev R, Spiegel S, Hla T (1998). Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. *Science* **279**:1552-1555

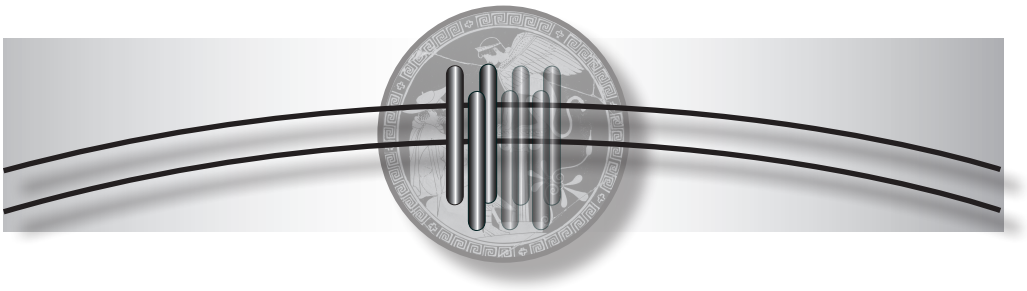
Liu Y, Wada R, Yamashita T, Mi Y, Deng CX, Hobson JP, Rosenfeldt HM, Nava VE, Chae SS, Lee MJ, Liu CH, Hla T, Spiegel S, Proia RL (2000). Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. *J Clin Invest* **106**:951-961

Mulvany MJ, Halpern W (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ Res* **41**:19-26

Spiegel S, Milstien S (2003). Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat Rev Mol Cell Biol* **4**:397-407

Watterson KR, Ratz PH, Spiegel S (2005). The role of sphingosine-1-phosphate in smooth muscle contraction. *Cell Signa*l **17:**289–298

Yatomi Y, Igarashi Y, Yang L, Hisano N, Qi R, Asazuma N, Satoh K, Ozaki Y, Kume S (1997). Sphingosine 1-phosphate, a bioactive sphingolipid abundantly stored in platelets, is a normal constituent of human plasma and serum. *J Biochem (Tokyo)* **121**:969-973



Validation of a rapid, non-radioactive method to quantify internalization of G-protein coupled receptors

Chapter 3

Validation of a rapid, non-radioactive method to quantify internalization of G-protein coupled receptors

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Abstract

Agonist exposure can cause internalization of G-protein coupled receptors (GPCRs), which may be a part of desensitization but also of cellular signaling. Previous methods to study internalization have been tedious or only poorly quantitative. Therefore, we have developed and validated a quantitative method using a sphingosine-1-phosphate (S1P) receptor as a model. Due to a lack of suitable binding studies, it has been difficult to study S1P receptor internalization. Using a N-terminal HisG-tag, $S1P_1$ receptors on the cell membrane can be visualized via immunocytochemistry with a specific anti-HisG antibody. S1P-induced internalization was concentration dependent and was quantified using a microplate reader, detecting either absorbance, a fluorescent or luminescent signal, depending on the antibodies used. Among those, the fluorescence detection method was the most convenient to use. The relative ease of this method makes it suitable to measure a large number of data points, e.g. to compare the potency and efficacy of receptor ligands.

Introduction

When chronically exposed to agonist, G-protein coupled receptors (GPCRs) can undergo desensitization, i.e. the response to a given level of agonist exposure declines and/or more agonist is required to maintain the same response. Various mechanisms can be involved in desensitization, some of which directly affect receptor responsiveness. One of these mechanisms involves phosphorylation of activated receptors by GPCR kinases (GRKs). This enables binding of arrestins to the receptor, leading to uncoupling from the G-protein and hence impaired signaling. Another, not necessarily mutually exclusive mechanism of desensitization is internalization of the receptor, which can be either homologous (due to activation of the receptor) or heterologous (due to activation of other receptors or to other stimuli). Receptor internalization can be arrestin-dependent or independent (Bünemann & Hosey, 1999) and involves translocation of the receptor from the cell membrane to the inside of the cell. There it is entrapped inside clathrin-coated pits and can undergo either degradation or resensitization.

Several approaches have been used in the past to detect and quantify GPCR internalization. One classical method involves homogenization of tissue or cells, differential centrifugation to yield a plasma membrane and a microsomal fraction followed by electrophoresis and autoradiographical analyses of the receptor of interest in both fractions (Simpson et al., 1984). Another classical method involves labeling of all receptors in intact cells with a lipophilic radioligand and competition by a hydrophilic ligand, which will replace the radioligand from sites at the cell surface but not from those entrapped intracellularly (Staehelin, 1983).

More recently, genetic approaches have been used. An example is the use of a C-terminal green fluorescent protein (GFP) (Barak et al., 1997; Kallal et al., 1998) to monitor receptor localization after ligand stimulation, sometimes combined with an algorithm to discriminate between internalized and membrane bound receptors (Conway et al., 1999; Schlag et al., 2004; Hirasawa et al., 2005; Fukunaga et al., 2006). This latter method uses a cell-based fluorescence imaging system ArrayScanII, where the amount of internalized receptor/GFP spots are counted and normalized against the number of nuclei stained with Hoechst. However, a GFP-, or other fluorescent tag is relatively large (200-300 amino acids) and can influence receptor function (McLean & Milligan, 2000). Other methods use a combination of techniques, like quantitative flow cytometry analysis of the cell surface receptors and immunocytochemical confocal microscopy analysis to visualize the subcellular

localization of the receptor of interest (Hirasawa et al., 1998).

Detailed quantitative studies of internalization are of interest as this process can be the mechanism of underlying physiological and pharmacological effects. For example, receptor internalization is thought to underlie the modulation of immune function by sphingosine-1-phosphate (S1P) (Matloubian et al., 2004). This also appears to be the mechanism of action of the immunosuppressive drug FTY720 (fingolimod) (Chiba et al., 2006; LaMontagne et al., 2006), which is undergoing clinical trials for the treatment of multiple sclerosis and previously also for renal graft rejection (Budde et al., 2002; Kappos et al., 2006).

In our search to quantify internalization of membrane receptors in a fast and easy way, we have setup a method, which uses a N-terminal HisG (6xHis+Gly)-tagged $S1P_1$ receptor. Since the HisG-tag is N-terminal, it is extracellular. Therefore, when immunocytochemistry is performed without permeabilization, only membrane-bound, tagged receptors will be detected. This way, internalization is quantified as a difference in membrane-fluorescence between unstimulated and agonist-stimulated cells.

The use of small, N-terminal tags has been described before (Von Zastrow et al., 1993; Wozniak & Limbird, 1996; Daunt et al., 1997; Vicentic et al., 2002; Miller, 2004). Of these, Daunt et al., 1997 described a method where cells were plated onto 24-well tissue culture dishes. After stimulations with an agonist, incubations with 1st and 2nd antibodies followed. Measurements were done after adding alkaline phosphatase substrate, resulting in a colorimetric reading. In this paper, we now describe the validation of a similar method by testing various antibody combinations and detection methods to quantify internalization of N-terminal HisG-tagged receptors. This resulted in immunocytochemical method using a specific anti-HisG 1st antibody combined with a fluorescent 2nd antibody (AlexaFluor® 488) as our preferred choice. The signal can either be visualized under the fluorescence microscope or measured in a fluorescence microplate reader. The latter results in quantitative measurement of the decrease in signal, caused by receptor internalization, in a direct, sensitive and fast way.

Materials & Methods

Materials

pcDNA3.1 containing the entire coding region of the human $S1P_1$ receptor was purchased from UMR cDNA Resource Center (Rolla, MO, USA).

Cell culture media, hygromycine B, Lipofectamine™2000, pcDNA3.1/His C, pOG44, CHO-FlpIn cells, Alexa Fluor® 488 goat anti-mouse (IgG), anti-HisG (mouse monoclonal IgG2a), anti-HisG-horse radish peroxidase (HRP) (mouse monoclonal IgG2a), pcDNA5/FRT/TO, NuPAGE® System, NuPAGE® Novex 4-12% Bis-Tris gel, MES running buffer, NuPAGE® transfer buffer and Invitrolon™ PVDF blotting membranes were obtained from Invitrogen (Breda, The Netherlands). BCA™ Protein Assay Kit was obtained from Pierce (Etten-Leur, The Netherlands). Formaldehyde solution was obtained from Sigma (Zwijndrecht, The Netherlands). Restriction enzymes (BamHI, XhoI, EcoRI and HindIII) were obtained from Fermentas Life Sciences (St. Leon-Rot, Germany). Black, clear bottom 96 well plates were obtained from Greiner Bio One (Alphen aan den Rijn, The Netherlands). S1P was obtained from Avanti-Polar Lipids, Inc (via Instruchemie B.V., Delfzijl, The Netherlands). Chamber slides were obtained from Nunc (Amsterdam, The Netherlands). BM Chemiluminescence Blotting Substrate [POD] and ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) were obtained from Roche (Mannheim, Germany). Hyperfilm ECL was obtained from Amersham Biosciences (Diegem, Belgium). SEW2871 (5-(4-Phenyl-5-trifluoromethylthiophen-2-yl)-3-(3-trifluoromethylphenyl)-1,2,4-oxadiazole) was obtained from Calbiochem (via VWR, Amsterdam, The Netherlands). Goat anti-mouse-HRP was obtained from Jackson ImmunoResearch Europe Ltd. (via Sanbio B.V.,Uden, The Netherlands). RIPA buffer was obtained from Boston bio products (Worcester, UK). Halt protease inhibitor cocktail was obtained from Pierce (via Perbio Science Nederland B.V. Etten-Leur, The Netherlands).

Molecular Cloning & Transfection

An N-terminal HisG-tag was added to the $S1P_1$ receptor via cloning into pcDNA3.1/HisC using BamHI&XhoI. A second cloning step was done using HindIII&XhoI to clone the HisG-tagged $S1P_1$ receptor into the expression vector pcDNA5/FRT/TO. Transfection of plasmids into CHO-FlpIn cells and cell culture was done as described previously (Chapter 2).

Western blot

Cells were washed and scraped in PBS, spun down, and lysed in RIPA buffer with protease inhibitor. Protein concentration was determined using the BCA kit according to the manufacturer's protocol. Ten µg of protein were loaded onto a 4-12 % NuPAGE® Bis-Tris Gel. Electrophoresis was carried out at 200 V for 40 min in NuPAGE® MES running buffer. Protein was transferred to Invitrolon™ PVDF blotting membranes at 30 V for 1 hour. The membrane was blocked for 1 hour at room-temperature in 50 mg/ml non-fat dry milk in PBS, followed by overnight incubation at 4 °C with a dilution of 1:5000 for mouse anti-HisG. After washing with PBS-Tween (0.1 %), the membrane was incubated with a 1:20,000 dilution of goat anti-mouse-HRP IgG at room-temperature. Detection was done using BM Chemiluminescence Blotting Substrate and Hyperfilm ECL.

Immunocytochemistry

Cells (40,000 cells/well) were plated on day one in 96 wells black, clear bottom plates or 8-well chamber slides. The next day, medium was changed to serum free medium. After incubation overnight, cells were stimulated with the indicated ligand in serum free medium for 30 minutes at 37 °C, unless otherwise indicated. The stimulation solutions were removed and cells were fixed by applying 4 % v/v formaldehyde in PBS for 15 minutes at room-temperature. After washing 3 times with PBS, cells were incubated with a 1st antibody for 1 hour at room-temperature. Cells were washed again 3 times with PBS and if necessary incubated with a 2nd antibody for 1 hour at room-temperature. Cells were washed 3 times with PBS followed by measurements on a platereader (Victor 2, Wallac, Perkin Elmer). The following antibody combinations were used: mouse anti-HisG 1:200 with AlexaFluor® 488 goat anti-mouse 1:500, mouse anti-HisG-HRP 1:200 and mouse anti-HisG 1:200 with goat anti-mouse-HRP 1:500. In case an HRP-bound antibody was used, either ABTS solution (50 µg/ml) or BM Chemiluminescence Blotting Substrate was added to generate an absorbance or luminescence signal respectively. For the absorbance signal a 405 nm filter was used and for the fluorescent signal a 490 nm excitation, and a 535 nm emission filter were used.

Data analysis

Concentration response curves were analyzed by fitting sigmoidal functions to the experimental data using Prism 4 (Graphpad Software, Inc., San Diego, CA, USA). Data are expressed as means ± s.e.m. Differences between groups were compared using a one way ANOVA with a Dunnett's correction or a Student's t-test where appropriate. $P<0.05$ was considered significant.

Results

Characterization of the HisG-tagged $S1P_1$ receptor

The presence of HisG-$S1P_1$ receptor in stably transfected CHO-FlpIn cells was confirmed in Western blot analysis as a band of approximately 50 kDa (Figure 1). The HisG-$S1P_1$ receptor was also visualized in the cell membrane by fluorescence microscopy using AlexaFluor® 488 goat anti-mouse as a secondary antibody (Figure 2). CHO-FlpIn cells stably expressing the untagged $S1P_1$ receptor were used as a negative control for both detection methods (Figures 1 and 2). Addition of the N-terminal HisG-tag to the $S1P_1$ receptor did not influence signaling via this receptor as it did not significantly alter the potency of S1P to inhibit forskolin-stimulated cAMP accumulation (pEC_{50} $S1P_1$ 9.0 ± 0.1 vs HisG-$S1P_1$ 8.9 ± 0.1; n=6), measured as described previously (Chapter 2).

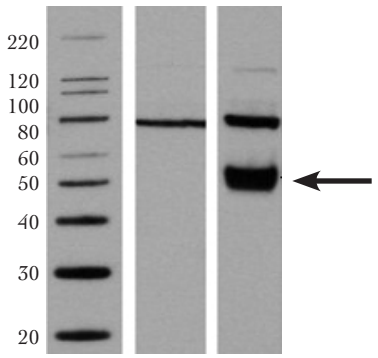
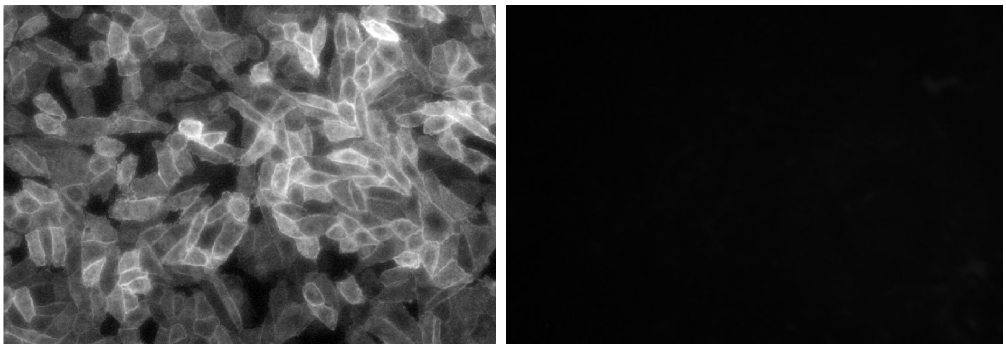


Figure 1. Western blot of CHO-FlpIn cells stably expressing the $S1P_1$ receptor (lane 1) and HisG-$S1P_1$ receptor (lane 2) using a mouse anti-HisG 1st antibody and a goat anti-mouse HRP 2nd antibody. HisG-$S1P_1$ is detected at approximately 50 kDa as indicated by the arrow. The band at 80 kDa is nonspecific. The blot shown is from a typical experiment out of 4.



A B

Figure 2. Fluorescence microscope pictures of CHO-FlpIn cells stably expressing the HisG-S1P$_1$ receptor(A) and the S1P$_1$ receptor (B) using a mouse anti-HisG 1st antibody and AlexaFluor® 488 goat anti-mouse 2nd antibody. Images shown are from one typical experiment out of 9.

Internalization of the S1P$_1$ receptor

A 30 min incubation with 1 µM S1P or 10 µM of the selective S1P$_1$ agonist SEW2871 (Sanna et al., 2004) at 37 °C decreased fluorescence (Figure 3A,C,E). No such decrease of fluorescence was seen when cells were incubated with either agonist at 4 °C (Figure 3B,D,F). S1P concentration-dependently internalized the HisG-S1P$_1$ receptor as visualized with a fluorescence microscope (Figure 4). Such decrease in fluorescence was also measured using a fluorescence microplate reader. The fluorescent signal of cells stably expressing the HisG-S1P$_1$ receptor was significantly higher than for cells stably expressing the untagged S1P$_1$ receptor. Stimulation with S1P (1 µM) for 30 min significantly decreased this signal (Figure 5).

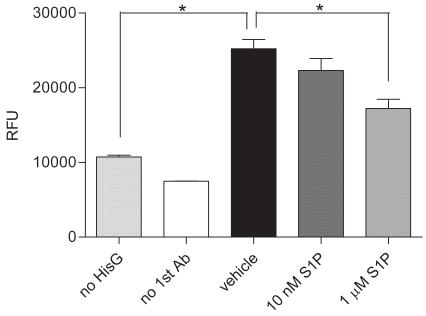


Figure 5. Quantitative measurements of cells stably expressing HisG-S1P$_1$ using a mouse anti-HisG 1st antibody and AlexaFluor® 488 goat anti-mouse 2nd antibody. Cells were stimulated at 37 °C for 30 min at the indicated S1P concentrations. The first bar represents cells stably expressing the S1P$_1$ receptor without a HisG-tag. The second bar represents cells stably expressing HisG-S1P$_1$ incubated without the 1st antibody. Statistical significance was shown between 'vehicle' and '1 µM S1P' as well as between 'no HisG' and 'vehicle'. Data are shown as relative fluorescent units (RFU) ± s.e.m. (n=3).

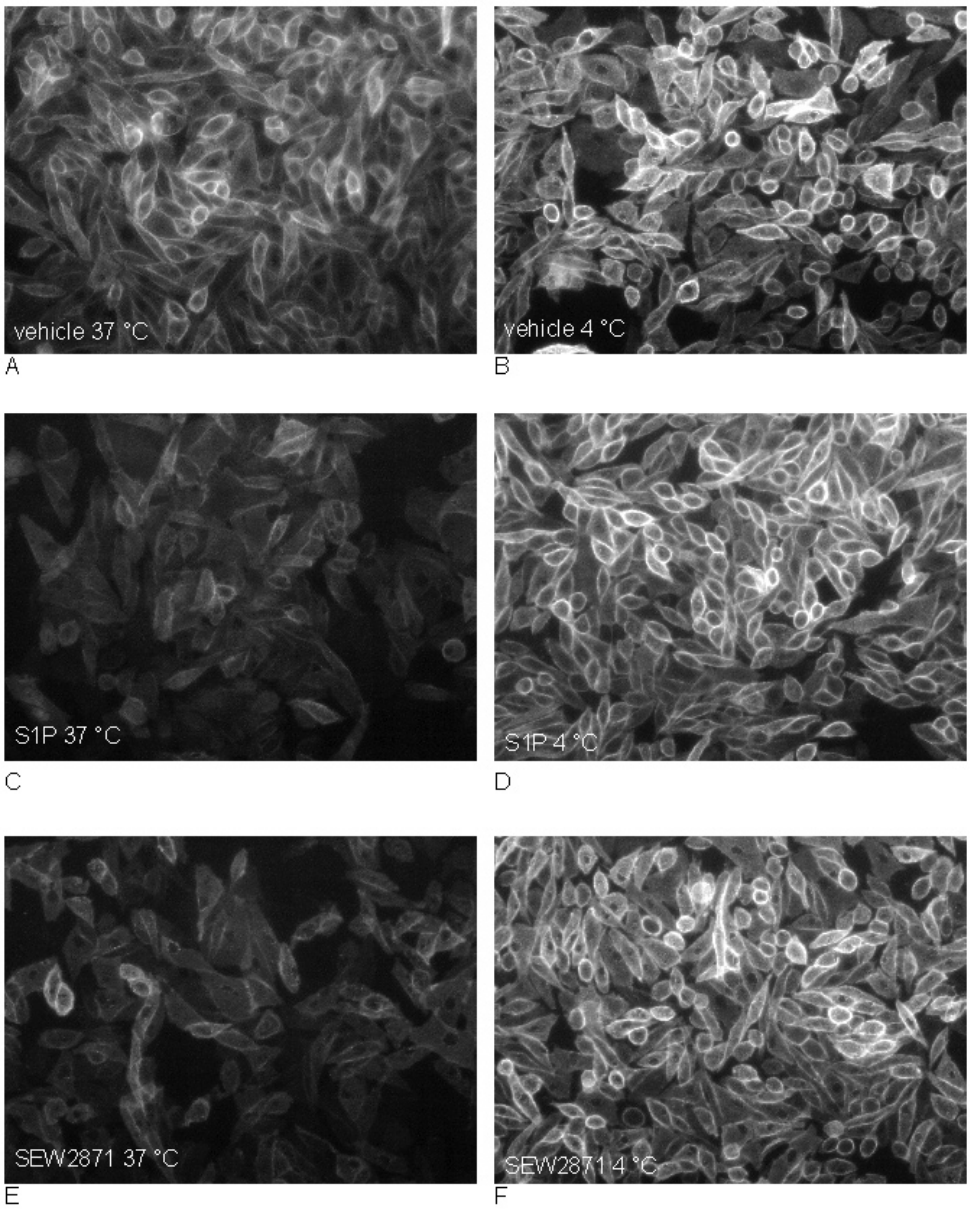


Figure 3. Fluorescence microscope pictures of CHO-FlpIn cells stably expressing the HisG-S1P$_1$ using a mouse anti-HisG 1st antibody and AlexaFluor® 488 goat anti-mouse 2nd antibody. Cells were stimulated for 30 min with 1 µM S1P (C,D) or 10 µM SEW2871 (E,F) or vehicle (A,B) at either 37 °C (left panel) or 4 °C (right panel). Images shown are from one typical experiment out of 4.

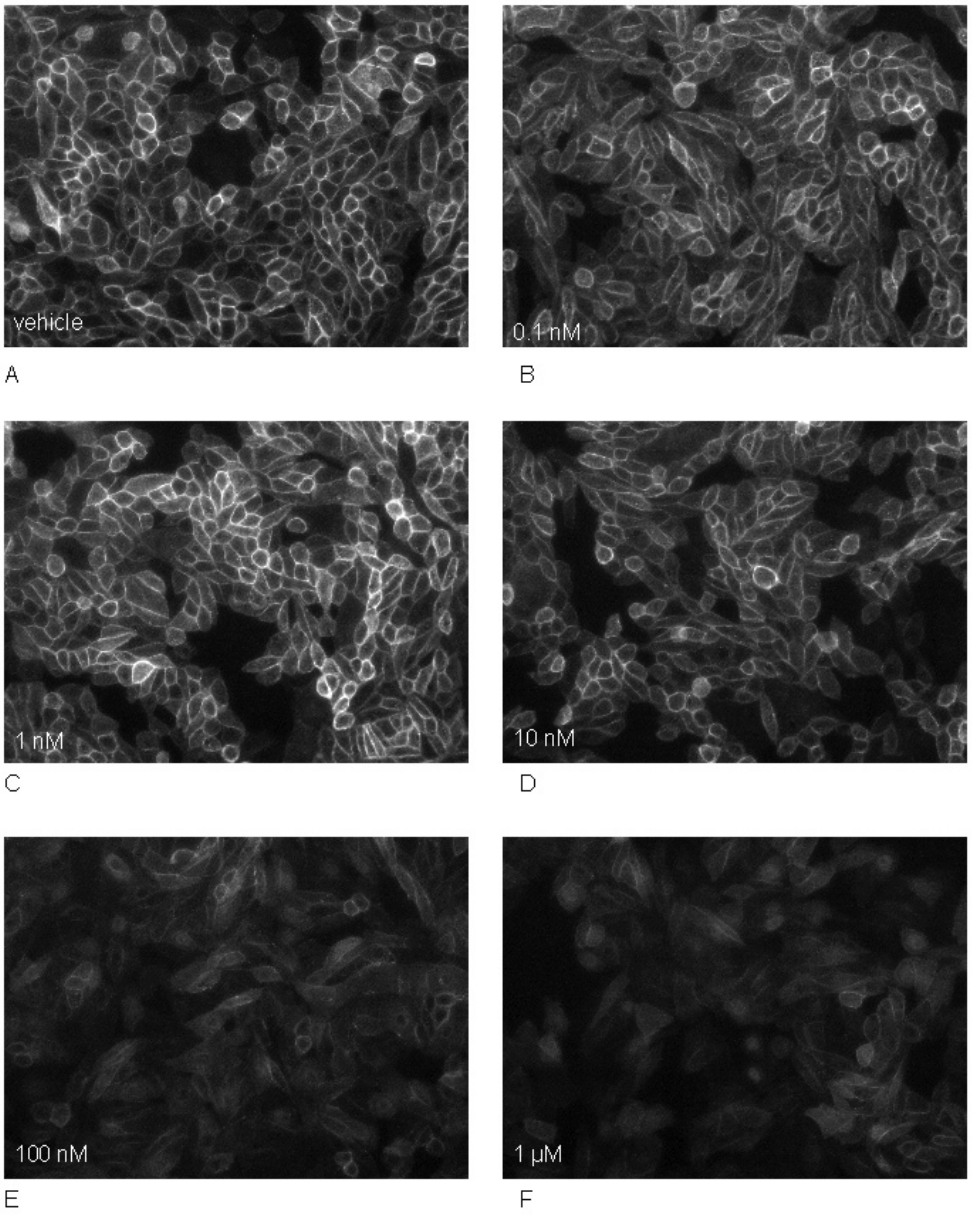


Figure 4. Fluorescence microscope pictures of CHO-FlpIn cells stably expressing the HisG-S1P$_1$ receptor using a mouse anti-HisG 1st antibody and AlexaFluor® 488 goat anti-mouse 2nd antibody. Cells were stimulated with S1P at the indicated concentrations (vehicle (A), 0.1 nM (B), 1 nm (C), 10 nM (D), 100 nM (E) or 1 µM (F)) at 37 °C for 30 min. Images shown are from one typical experiment out of 4.

Choice of detection method

Instead of the antibody combination mouse anti-HisG and AlexaFluor® 488 goat anti-mouse (Figure 6A), other combinations were tested to optimize the method. The use of a 1st antibody mouse anti-HisG-HRP (Figure 6B), or a 1st antibody mouse anti-HisG with a 2nd antibody goat anti-mouse-HRP (Figure 6C) combined with the BM Chemiluminescence Blotting Substrate (POD) resulted in a luminescence signal which proved very sensitive. However, the signal was very unstable over time, which required addition of the substrate by the microplate reader. The combination of a 1st antibody mouse anti-HisG-HRP (Figure 6D), or a 1st antibody mouse anti-HisG with a 2nd antibody goat anti-mouse-HRP (Figure 6E) combined with ABTS resulted in an absorbance signal. This signal however, had less

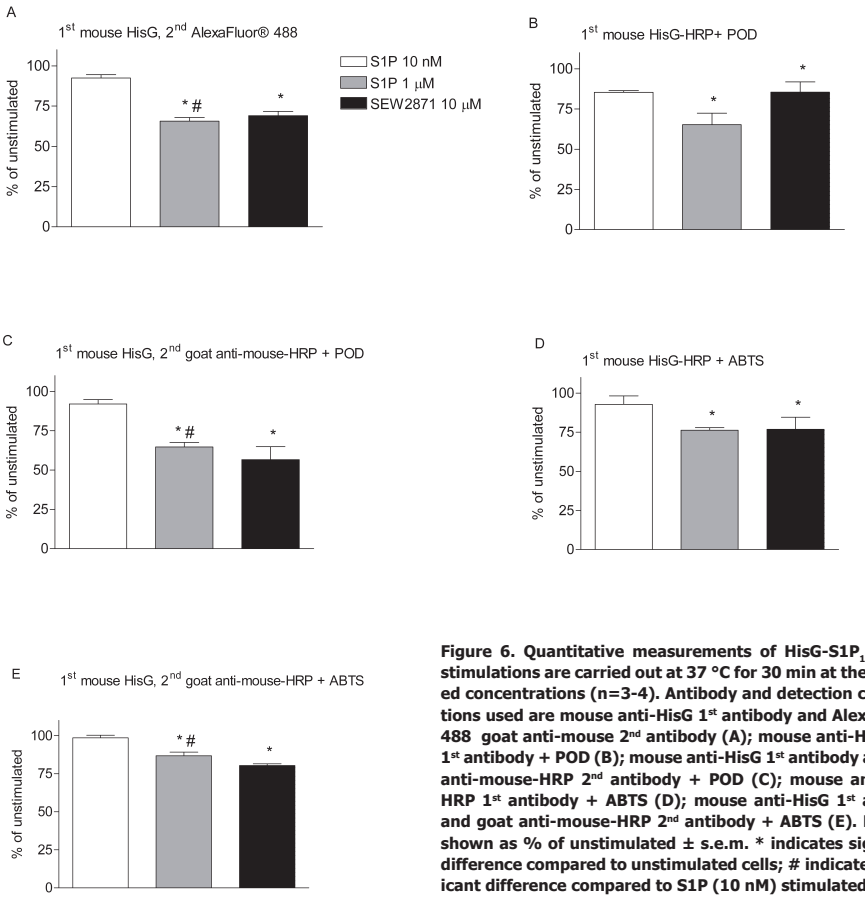


Figure 6. Quantitative measurements of HisG-S1P$_1$. Ligand stimulations are carried out at 37 °C for 30 min at the indicated concentrations (n=3-4). Antibody and detection combinations used are mouse anti-HisG 1st antibody and AlexaFluor® 488 goat anti-mouse 2nd antibody (A); mouse anti-HisG-HRP 1st antibody + POD (B); mouse anti-HisG 1st antibody and goat anti-mouse-HRP 2nd antibody + POD (C); mouse anti-HisG-HRP 1st antibody + ABTS (D); mouse anti-HisG 1st antibody and goat anti-mouse-HRP 2nd antibody + ABTS (E). Data are shown as % of unstimulated ± s.e.m. * indicates significant difference compared to unstimulated cells; # indicates significant difference compared to S1P (10 nM) stimulated cells.

sensitivity compared to the other two detection methods. The use of a 1st antibody mouse anti-HisG-HRP resulted in a loss of sensitivity compared to the combination of a 1st antibody mouse anti-HisG with a 2nd antibody goat anti-mouse-HRP.
When the combination of a 1st antibody mouse anti-HisG and a 2nd antibody AlexaFluor® 488 goat anti-mouse was used, we measured a concentration-dependent internalization of HisG-$S1P_1$ after stimulation with either S1P or SEW2871 (pEC_{50} S1P = 7.4 ± 0.1 (n=8) and pEC_{50} SEW2871 = 5.7 ± 0.2 (n=3) respectively, Figure 7).

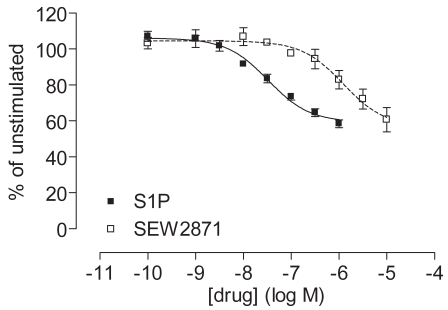


Figure 7. Effect of increasing concentrations of S1P (closed squares) or SEW2871 (open squares) on CHO-FlpIn cells stably expressing the HisG-$S1P_1$ receptor, using a mouse anti-HisG 1st antibody and AlexaFluor® 488 goat anti-mouse 2nd antibody. Stimulations were carried out at 37 °C for 30 min. The data shown represent the mean ± s.e.m. of 6 independent experiments performed in triplicate.

Discussion

Previously reported methods to study the internalization of GPCRs were very labor-intensive, required specific equipment, added bulky tags and/or were poorly quantitative. We have developed and validated a method which uses stably transfected cells expressing a receptor with a minor modification only, i.e. an N-terminal HisG-tag, which did not influence receptor signaling. Several antibody combinations and detection methods were tested. The luminescent approach was very sensitive, but highly dependent on strict adherence to a specific time interval between substrate addition and actual signal measurement. The absorbant signal obtained using another detection substrate resulted in a loss of sensitivity. The antibody combination of a 1st HisG specific antibody and a 2nd AlexaFluor® 488 fluorescent antibody proved the most convenient, resulting in a sensitive and stable signal. Compared to previously published methods using a similar approach (Von Zastrow et al., 1993; Wozniak & Limbird, 1996; Daunt et al., 1997; Vicentic et al., 2002),

this immunocytochemical detection of the N-terminal HisG-tag is suitable to assess a large number of samples e.g. to construct detailed concentration response curves and/or compare multiple ligands. In this regard, we showed that S1P was approximately 50 times more potent than SEW2871 in internalising the HisG-$S1P_1$ receptor. This is in good agreement with the previously reported potency difference between the two agonists for activating the receptor (Sanna et al., 2004). Since comparable methods have been described for adrenergic receptors as mentioned above (Daunt et al., 1997; Vicentic et al., 2002; Wozniak & Limbird, 1996; Von Zastrow et al., 1993), it is likely that the method we describe here is also suitable for receptors other than our model receptor, the $S1P_1$ receptor.

While our technique does not allow following internalization in real time, the ability to process a large number of samples compensates for that by studying multiple time points. The only limitation to our method is the inability to measure internalization of endogenous receptors. However, this would be possible when specific receptor antibodies are available, which is not yet the case for our model receptor.

In conclusion, we have validated a method to quantitatively measure receptor internalization in which we use an N-terminal HisG-tag combined with immunocytochemistry. It proves to be sensitive enough to discriminate between different receptor ligands in a fast and non-radioactive way.

References

Barak LS, Ferguson SSG, Zhang J, Martenson C, Meyer T, Caron MG (1997) Internal trafficking and surface mobility of a intact β2-adrenergic receptor-green fluorescent protein conjugate. *Mol Pharmacol* **51**: 177-184

Budde K, Schmouder RL, Brunkhorst R, Nashan B, Lucker PW, Mayer T, Choudhury S, Skerjanec A, Kraus G, Neumayer HH (2002) First human trial of FTY720, a novel immunomodulator, in stable renal transplant patients. *J Am Soc Nephrol* **13**:1073-1083

Bünemann M, Hosey MM (1999) G-protein coupled receptor kinases as modulators of G-protein signaling. *J Physiol* **517**: 5-23

Chiba K, Matsuyuki H, Maeda Y, Sugahara K (2006) Role of sphingosine 1-phosphate receptor type 1 in lymphocyte egress from secondary lymphoid tissues and thymus. *Cell Mol Immunol* **3**:11-19

Conway BR, Minor LK, Xu JZ, Gunnet JW, DeBiasio R, D'Andrea MR, Rubin R, DeBiasio R, Giuliano K, DeBiasio L, Demarest KT (1999) Quantification of G-protein coupled receptor internalization using G-protein coupled receptor-green fluorescent protein conjugates with the ArrayScantrade mark high-content screening system. *J Biomol Screen* **4**:75-86

Daunt DA, Hurt C, Hein L, Kallio J, Feng F, Kobilka BK (1997) Subtype-specific intracellular trafficking of α2-adrenergic receptors. *Mol Pharmacol* **51**:711-720

Fukunaga S., Setoguchi S., Hirasawa A., Tsujimoto G. (2006) Monitoring ligand-mediated internalization of G protein-coupled receptor as a novel pharmacological approach. *Life Sci* **80**:17-23

Hirasawa A, Awaji T, Sugawara T, Tsujimoto A, Tsujimoto G (1998) Differential mechanism for the cell surface sorting and agonist-promoted internalization of the α1B-adrenoceptor. *Br J Pharmacol* **124**:55-62

Hirasawa A, Tsumaya K, Awaji T, Katsuma S, Adachi T, Yamada M, Sugimoto Y, Miyazaki S, Tsujimoto G. (2005) Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. *Nat Med* **11**:90-94

Kallal L, Gagnon AW, Penn RB, Benovic JL (1998) Visualization of agonist-induced sequestration and down-regulation of a green fluorescent protein-tagged β2-adrenergic receptor. *J Biol Chem* **273**:322-328

Kappos L, Antel J, Comi G, Montalban X, O'Connor P, Polman CH, Haas T, Korn AA, Karlsson G, Radue EW (2006) Oral fingolimod (FTY720) for relapsing multiple sclerosis. *N Engl J Med* **355**:1124-1140

LaMontagne K, Littlewood-Evans A, Schnell C, O'Reilly T, Wyder L, Sanchez T, Probst B, Butler J, Wood A, Liau G, Billy E, Theuer A, Hla T, Wood J (2006) Antagonism of sphingosine-1-phosphate receptors by FTY720 inhibits angiogenesis and tumor vascularization. *Cancer Res* **66**:221-231

Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, Brinkmann V, Allende ML, Proia RL, Cyster JG (2004) Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature* **427**:355-360

McLean AJ, Milligan G (2000) Ligand regulation of green fluorescent protein-tagged forms of the human β1- and β2-adrenoceptors; comparisons with the unmodified receptors. *Br J Pharmacol* **130**:1825-1832

Miller JW (2004) Tracking G protein-coupled receptor trafficking using Odyssey imaging. Published August, by LI-COR Biosciences (http://www.licor.com/bio/PDF/Miller_GPCR.pdf accession date Jan 12 2007)

Sanna MG, Liao J, Jo E, Alfonso C, Ahn MY, Peterson MS, Webb B, Lefebvre S, Chun J, Gray N, Rosen H (2004) Sphingosine 1-phosphate (S1P) receptor subtypes S1P1 and S1P3, respectively, regulate lymphocyte recirculation and heart rate. *J Biol Chem* **279**:13839–13848

Schlag BD, Lou Z, Fennell M, Dunlop J. (2004) Ligand dependency of 5-hydroxytryptamine 2C receptor internalization. *J Pharmacol Exp Ther* **310**: 865-870

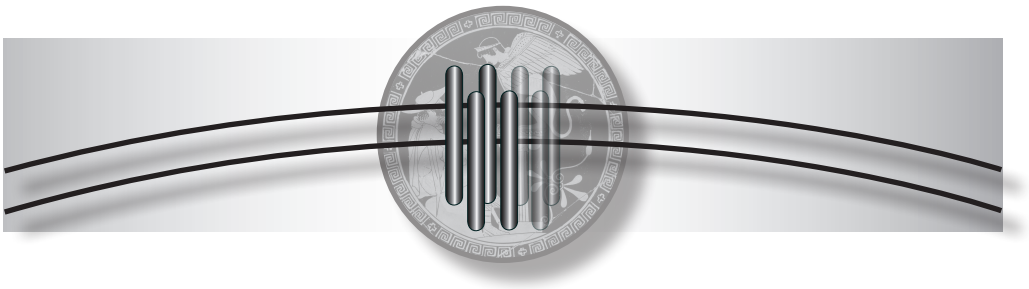
Simpson IA, Hedo JA, Cushman SW (1984) Insulin-induced internalization of the insulin receptor in the isolated rat adipose cell. Detection of both major receptor subunits following their biosynthetic labeling in culture. *Diabetes* **33**:13-18

Staehelin M, Simons P, Jaeggi K, Wigger N (1983) CGP-12177. A hydrophilic beta-adrenergic receptor radioligand reveals high affinity binding of agonists to intact cells. *J Biol Chem* **258**:3496-3502

Vicentic A, Robeva A, Rogge G, Uberti M, Minneman KP (2002) Biochemistry and pharmacology of epitope-tagged α1-adrenergic receptor subtypes. *J Pharmacol Exp Ther* **302**:58-65

Wozniak M, Limbird LE (1996) The three α2-adrenergic receptor subtypes achieve basolateral localization in Madin-Darby canina kidney II cells via different targeting mechanisms. *J Biol Chem* **271**:5017-5024

Von Zastrow M, Link R, Daunt D, Barsch G, Kobilka B (1993) Subtype-specific differences in the intracellular sorting of G protein-coupled receptors. *J Biol Chem* **268**:763-766



Pharmacological and biochemical characterization of the vasoactive properties of sphingosine-1-phosphate receptors in rat aorta

Chapter 4

Pharmacological and biochemical characterization of the vasoactive properties of sphingosine-1-phosphate receptors in rat aorta

Manuscript submitted

Maikel Jongsma, Marie-Jeanne Mathy, Pieter B. van Loenen, Najat Hajji, Martin C. Michel, Stephan L.M. Peters & Astrid E. Alewijnse

Abstract

The bioactive sphingolipid sphingosine-1-phosphate (S1P) exerts diverse cellular effects via a family of five specific G-protein coupled receptors, named $S1P_{1\text{-}5}$.
In the cardiovascular system, the $S1P_1$, $S1P_2$ and $S1P_3$ receptors are differentially expressed in both vascular smooth muscle cells (VSMCs) and endothelial cells (ECs). The vasoactive effects of S1P are, therefore, a result of the balance between expression of these receptors on either cell type. We have explored this balance in the rat aorta using new synthetic S1P receptor ligands combined with biochemical methods. In intact isolated rat aortic rings, agonists at the $S1P_1$ and/or $S1P_3$ receptor (FTY720, FTY720-P, SEW2871 and VPC23153) did not induce vasoconstriction, but rather endothelium dependent vasorelaxation of pre-contracted preparations. In rat aortic ECs only the G_i-coupled $S1P_1$ receptor was highly expressed. In these cells, S1P, FTY720, FTY720-P and SEW2871 inhibited cAMP accumulation via $S1P_1$. Additionally, VPC23019 competitively antagonized the FTY720-P-induced inhibition of the cAMP accumulation. VSMCs showed low mRNA expression of all three S1P receptors. S1P-induced elevation of $[Ca^{2+}]_i$ in these cells was observed via predominantly the $S1P_2$ receptor, since JTE-013 partially inhibited this response. In addition, minor $S1P_1$ and/or $S1P_3$-mediated effects on cAMP accumulation were observed in VSMCs. In conclusion, by using newly available pharmacological tools, we have shown vasodilation-promoting $S1P_1$ receptor-mediated effects in ECs and vasoconstricting-promoting $S1P_2$ receptor-mediated effects in VSMCs, which for most compounds results in vasorelaxation in rat aorta.

Introduction

Sphingosine-1-phosphate (S1P) is an important lipid mediator generated from membrane lipids upon cell activation. S1P can be synthesized from sphingomyelin, a major constituent of most cellular membranes, via the action of several enzymes (for a review see Hannun et al., 2001). Besides local production in the vessel wall (Mulders et al., 2006), S1P may reach smooth muscle or endothelial cells via the bloodstream, since S1P is a constituent of high-density lipoproteins (Murata et al., 2000), can be released from activated platelets (Yatomi et al., 2000) and is a major constituent of erythrocytes (Pappu et al., 2007). S1P has profound effects on vascular cells as it shows anti-apoptotic and growth-inducing properties on both smooth muscle and endothelial cells, but also has vasoactive properties (Peters & Alewijnse, 2007). Although intracellular targets have been suggested (Payne et al., 2002), most of the aforementioned effects are mediated via specific S1P receptors present on the cell membrane. Until now, five different S1P receptor subtypes have been described, named $S1P_{1\text{-}5}$ of which in the cardiovascular system the $S1P_1$, $S1P_2$ and $S1P_3$ receptors are the predominant S1P receptors (Kluk & Hla, 2001).

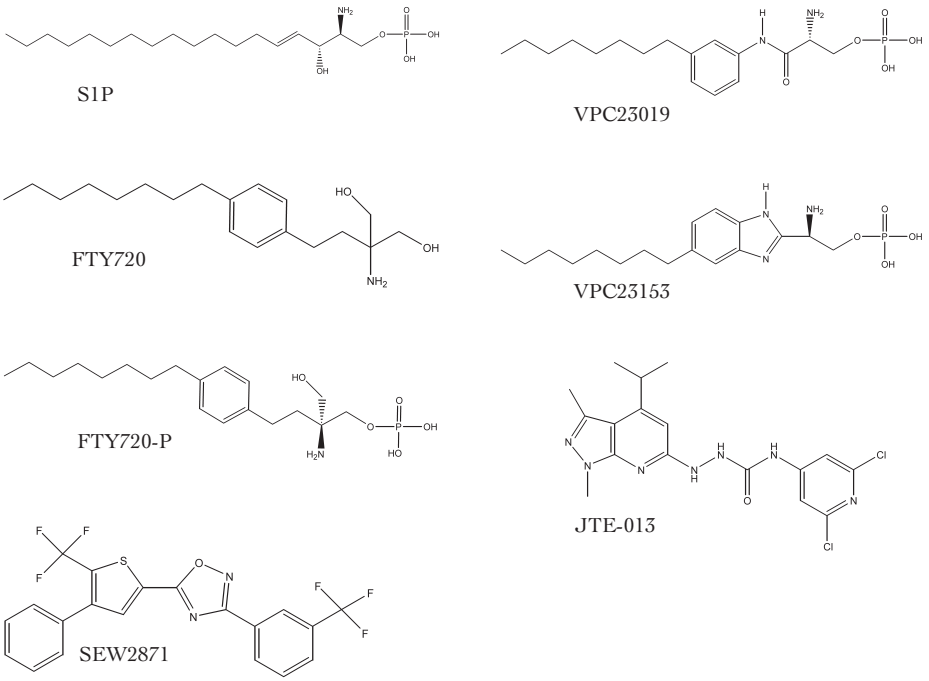


Figure 1. Chemical structure of ligands for the $S1P_1$, $S1P_2$ and $S1P_3$ receptors.

S1P increases intracellular calcium in both vascular smooth muscle and endothelial cells via the activation of S1P receptors. Increases in intracellular calcium in vascular smooth muscle cells are linked to increased constriction. In contrast, a rise in intracellular calcium in the endothelial cell will induce NO production via activation of endothelial NO synthase (eNOS) resulting in vasodilation. Furthermore, activation of endothelial $S1P_1$ and $S1P_3$ receptors may lead to enhanced NO production via the activation of the PI3Kinase/Akt pathway and subsequent eNOS phosphorylation (Morales-Ruiz et al., 2001; Nofer et al., 2004; Mulders et al., 2006). The net effect of S1P on the vessel, therefore, depends on the relative expression of S1P receptor subtypes in smooth muscle and endothelial cells. Indeed, S1P has been shown to induce both, vasodilation and vasoconstriction depending, amongst others, on species, applied techniques, and vessel type used (Waeber et al., 2004; Hemmings, 2006). However, research up till now on the role of S1P and its receptors in the vascular system has been hampered by the sparse availability of specific receptor ligands.

Table 1. Overview of properties and pharmacological characteristics of ligands for the $S1P_1$, $S1P_2$ and $S1P_3$ receptors.
[1] Young and van Brocklyn, 2006; [2] Mandala et al., 2002 IC_{50} (binding); [3] Sanna et al., 2004: EC_{50} (GTPγS); [4.1] Davis et al., 2005: pK_B (GTPγS); [4.2] Davis et al., 2005: pK_i (binding); [5] Ohmori et al., 2003: IC_{50}.

Ligand	Properties	Pharmacological characteristics		
		$S1P_1$ (nM)	$S1P_2$ (nM)	$S1P_3$ (nM)
S1P	$S1P_{1\text{-}5}$ agonist	24.7[1] 0.47[2]	18.4[1] 0.31[2]	19.7[1] 0.17[2]
FTY720	-	300[2]	>10000[2]	>10000[2]
FTY720-P	$S1P_{1,3,4,5}$ agonist	0.21[2]	>10000[2]	5.0[2]
SEW2871	$S1P_1$ agonist	13[3]	>10000[3]	>10000[2]
VPC23153	$S1P_{1,3}$ agonist	no values available		
VPC23019	$S1P_{1,3}$ antagonist	32[4.1]	no effect	1047[4.1] 1175[4.2]
JTE-013	$S1P_2$ antagonist	13.8[4.2]	17[5]	no effect

Recently several agonists and antagonists of the $S1P_1$, $S1P_2$ and $S1P_3$ receptors have become available (Table 1, Figure 1) which makes it possible for the first time to pharmacologically characterize vascular S1P receptor function. This study was designed to use the newly available S1P receptor ligands to investigate vascular S1P receptor signaling in vitro by combining contractile effects in isolated rat aortas and designate these effects to specific S1P receptors in either smooth muscle or endothelial cells.

Materials and Methods

Materials

Medium 199, Hanks' Balanced Salt Solution (HBSS), DNAseI Amp grade and Trizol were obtained from Invitrogen (Breda, The Netherlands). Phosphate buffered saline (PBS), foetal calf serum (FCS), enzyme-free cell dissociation buffer and penicillin/streptomycin were obtained from Gibco (via Invitrogen). LANCE cAMP 384 kit and white 384-well optiplates were obtained from Perkin-Elmer (Zaventem, Belgium). S1P, (R)-Phosphoric acid mono-[2-amino-2-(3-octylphenylcarbamoyl)-ethyl] ester (VPC23019), (R)-Phosphoric acid mono-[2-amino-2-(6-octyl-1H-benzoimiazol-2-yl) ethyl] ester (VPC23153) and were obtained from Avanti-Polar Lipids Inc. (via Instruchemie B.V., Delfzijl, The Netherlands). Probenecid, HEPES, ethyleneglycoltetraacetate, Triton X-100, 3-isobutyl-1-methylxanthine, bovine serum albumin (regular and fatty-acid free), collagenase, elastase, gelatin, RNAlater, forskolin, sodium citrate, ATP, bovine serum albumine (BSA) and trypsin inhibitor were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Clear bottom black 96-well plates were obtained from Greiner Bio One (Alphen aan den Rijn, The Netherlands). Pluronic acid and Fluo-4 AM were obtained from Molecular Probes (via Invitrogen). qPCR primers were obtained from Biolegio (Nijmegen, The Netherlands). N-(2,6-dichloro-4-piridinyl)-2-[1,3-dimethyl-4-(1-methylethyl)-1H-pyrazolo[3,4-b]pyridin-6-yl]-hydrazinecarboxamide (JTE-013) was obtained from Tocris (Bristol, UK). Isopropanol, ethanol, and chloroform were obtained from Merck (Nottingham, UK). SYBR green supermix was obtained from Biorad Laboratories BV (Veenendaal, The Netherlands). Rat aortic endothelial cells (RAECs), attachment factor and rat endothelial cell growth medium were obtained from Cell Applications, Inc. (San Diego, CA). 2-Amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol (FTY720) was obtained from ITK Diagnostics BV (Uithoorn, the Netherlands). 2-Amino-2-[2-(4-octylphenyl) ethyl]-1,3-propanediol mono(dihydrogen phosphate) ester (FTY720-P) was synthesized according to previously described methods (Albert et al., 2005). 5-[4-Phenyl-5-(trifluoromethyl)-2-thienyl]-3-[3-(trifluoromethyl)phenyl]-1,2,4-oxadiazole (SEW2871) was obtained from Cayman Chemical Company (Ann Arbor, MI)

Contraction and relaxation studies

Male Wistar rats (Charles River, Maastricht, The Netherlands) weighing 240-280 grams were anesthetized with pentobarbital (100 mg/kg i.p.) The thoracic aorta

was isolated and placed in an oxygenated Tyrode's solution (118.5 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 0.025 mM Na_4EDTA, 1.8 mM $CaCl_2$, 1.2 mM KH_2PO_4, 5.5 mM glucose, 25 mM $NaHCO_3$) at room temperature and cleaned of superficial fat and loose connective tissue. Thereafter, the aorta was cut into rings of approximately 3 mm length that subsequently were mounted between two triangular stainless steel hooks in water jacketed (37 °C) organ baths containing Tyrode's solution. This was continuously gassed with a mixture of 95% O_2 / 5% CO_2. Isometric force of contraction was recorded on a Powerlab data acquisition system (AD Instruments, Bella Vista, NSW, Australia). The resting tension was set to 10 mN and the segments were allowed to equilibrate for at least 45 min. After this equilibration period the preparations were exposed thrice to a depolarizing 40 mM K^+ solution, with 30 min intervals. The presence of a functional endothelium was assessed by precontracting the segments with 1 µM phenylephrine, and a subsequent addition of 10 µM methacholine. Segments that had a relaxation less than 80% were excluded. For all relaxation studies, the vessel segments were preconstricted with 1 µM phenylephrine.

Vascular smooth muscle cell isolation and cell culture

Thoracic rat aorta was cleaned from fat-tissue and blood cloths and incubated while rotating for 30 min at 30 °C in digestive medium (medium 199 supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (basal medium), collagenase 1 mg/ml, elastase 42.5 µg/ml, BSA 1 mg/ml and trypsin inhibitor 0.5 mg/ml). The adventitia was removed, the aorta was washed in basal medium and resuspended in digestive medium. After 45 min incubation at 37 °C while rotating, the aorta was cut into small pieces, which were resuspended in digestive medium and incubated for 2.5 hours at 37 °C while rotating. Basal medium plus 20 % FCS was added and the mixture was spun down. The pellet was washed twice in basal medium plus 20 % FCS and transferred to a T25 tissue culture flask coated with 0.1 % gelatine in PBS. After reaching confluency, cells were passaged 1:1 to a T75 tissue culture flask coated with 0.1 % gelatine in PBS, in basal medium plus 10 % FCS. VSMCs were passaged 1:2 after reaching confluency in tissue culture flasks coated with 0.1 % gelatine in PBS, in basal medium plus 10 % FCS.

RAECs were cultured as described in the manufacturer's protocol.

Intracellular calcium measurements

Intracellular calcium measurements were performed as previously described (Chapter 2) with minor changes. In short, cells were plated in a black, clear bottom 96 wells plate at 40,000 cells/well for RAECs, or 10,000 cells/well for VSMCs. After one day growth, cells were serum starved overnight. Cells were then loaded for 1 hour with 4 µM Fluo-4 AM ester in HBSS containing 20 mM Hepes, 0.42 % v/v pluronic acid and 2.5 mM probenecid and incubated at 37 °C. After loading, cells were washed twice with HBSS containing 20 mM Hepes and 2.5 mM probenecid and incubated at 37 °C with HBSS containing 20 mM Hepes and 2.5 mM probenecid in the presence or absence of JTE-013 (1 µM or 10 µM) for 30 min. The fluorescence signal was measured at basal level, followed by ligand stimulation, Triton (5 % v/v) addition (resulting in F_{max}) and 250 mM EGTA addition (resulting in F_{min}). The $[Ca^{2+}]_i$ was calculated via the equation: $[Ca^{2+}]_i = Kd * ((F-F_{min})/(F_{max}-F))$. Kd being the dissociation constant of the binding of Fluo-4 to calcium (345 nM). The increase in $[Ca^{2+}]_i$ upon ligand stimulation was calculated as the difference between the $[Ca^{2+}]_i$ for the basal level and after adding a ligand.

cAMP assay

The LANCE™ cAMP 384 kit was used to determine cAMP concentrations according to the manufacturer's protocol. RAECs or VSMCs, after overnight serum-starvation, were detached from the surface using trypsin. Cells were washed once with HBSS and subsequently resuspended in buffer, containing HBSS with 0.05 % BSA (fatty-acid free) and 5 mM Hepes. Stimulation mixtures consisted of stimulation buffer with 3 µM forskolin, 0.5 mM 3-isobutyl-1-methylxanthine, with or without VPC23019 and the concentration response curve of the indicated compounds. All compounds were two times concentrated. Cells were added to the stimulation mixtures 1:1 in a 384 well optiplate at 2500 cells/well and stimulated for 1 hour. Detection of the cAMP formed during stimulation was done according to the manufacturer's protocol in a total volume of 20 µl. Measurements were done using a platereader (Victor 2, Wallac, Perkin Elmer) 3 hours after adding detection buffer and antibody mixture.

RNA isolation and cDNA synthesis

Cultured cells were detached from the cell-culture dish using Trizol. RNA was isolated as described below.

Total RNA was isolated according to the manufacturer's protocol with minor changes, using a second chloroform extraction to remove traces of phenol in the aqueous phase, a high salt solution (0.8 M sodium citrate, 1.2 M NaCl) together with isopropanol to precipitate RNA and a second wash of the RNA pellet with 75 % ethanol. RNA purity was verified on the Experion (Biorad Laboratories) and the RNA concentration was determined by spectrophotometry at the Nanodrop (Isogen Life Science). To eliminate genomic DNA contamination 1 µg of total RNA was treated with 1 µl DNase I, Amp Grade. cDNA was synthesized by reverse transcription using the iScript cDNA Synthesis kit according to the manufacturer's protocol. A control for the presence of genomic DNA, in which no cDNA was synthesized, was made for each sample. The cDNA of 1 µg RNA was diluted 1:50 for use in real-time quantitative PCR.

Primer design:

Oligonucleotide primers with a fluorophore near the 3´end containing a hairpin (non-capital letters) to render the fluorescence quenching capability, were designed using the D-LUX designer software (Invitrogen), based on sequences from the GenBank database: $S1P_1$ receptor (138 bp product), forward TTCCTGGTTCTGGCTGTGCT, reverse actccTTGAATTTGCCAGCGGAGtC; $S1P_2$ receptor (95 bp product), forward CCAACGGAGGCACTGACTAAT, reverse ATGTCTAGCCCTAAACTCGAGCC; $S1P_3$ receptor (79 bp product), forward caccagGCATCTTCACAGCCATTCTGGtG, reverse CTGCGGCTGCTGGACTTGAC; elongation factor-1 (EF-1, 96 bp product), forward GCAAGCCCATGTGTGTTGAA, reverse TGATGACACCCACAGCAACTG; p0 ribosomal protein (91 bp product), forward cacagaAGGGTCCTGGCTTTGTCTGtG, reverse CGCAAATGCAGATGGATCG. Each primer pair was tested for selectivity, sensitivity and PCR efficiency. Constitutively expressed EF-1 and p0 ribosomal protein were selected as endogenous controls to correct for potential variation in RNA loading.

Real-time quantitative PCR

Relative quantification of mRNA was performed on a MyiQ Single-Color Real-Time PCR Detection System (Biorad Laboratories) following the thermal protocol:

95 °C for 3 minutes to denature, 45 cycles at 95 °C for 10 s followed by 60 °C for 45 s for annealing and extension. The final reaction mixture of 15 µl consisted of diluted cDNA, 1x iQ SYBR Green Supermix, 200 nM forward primer and 200 nM reverse primer. All reactions were performed in duplicate in 96-well plates. Controls for genomic DNA were included for each cDNA sample and also a negative control containing only both primers and the iQ SYBR Green Supermix. All data were normalized to the expression of elongation factor 1 and ribosomal protein p0.

Data analysis

Concentration response curves were analyzed by fitting sigmoidal functions to the experimental data using Prism 4 (Graphpad Software, Inc., San Diego, CA, USA). Data are expressed as means ± s.e.m. Differences between groups were compared using a one way ANOVA with a Dunnett's correction or a Student's t-test where appropriate. $P<0.05$ was considered significant.

Results

Contraction and relaxation studies

None of the tested ligands affected the applied pretension of isolated rat aortic rings (data not shown), indicating that none of the compounds induces contraction in this preparation. Relaxant responses were measured in phenylephrine-preconstricted preparations. The phenylephrine (1 µM)-induced pre-constriction amounted to 17.5 ± 0.2 mN in the presence of endothelium (n=49) or 19.3 ± 0.5 mN (n=14) in the absence of endothelium. All compounds, with the exception of S1P, induced a concentration-dependent relaxation in the phenylephrine-preconstricted vascular preparations (Figure 1) with maximal effects of 39.4 ± 7.1 % (FTY720, n=5); 36.4 ± 4.0 % (FTY720-P, n=4); 18.0 ± 6.6 % (VPC23153, n=5) or 16.2 ± 4.6 % (SEW2871, n=4) ($p<0.05$). FTY720-P and its non-phosphorylated parent compound FTY720, induced comparable vasorelaxation, however FTY720-P-induced relaxation was much faster compared to FTY720 (1-2 min versus 8 min). Endothelium removal completely diminished relaxation for all compounds (Figure 2).

S1P receptor-mediated signaling in RAECs

Both, S1P and FTY720-P, potently inhibited the forskolin-induced cAMP accumulation (pEC_{50} = 7.8 ± 0.2 (n=10) and 8.5 ± 0.3 (n=5), respectively) in RAECs (Figure 3A) ($p<0.05$). FTY720 and SEW2871 also inhibited forskolin-induced cAMP accumula-

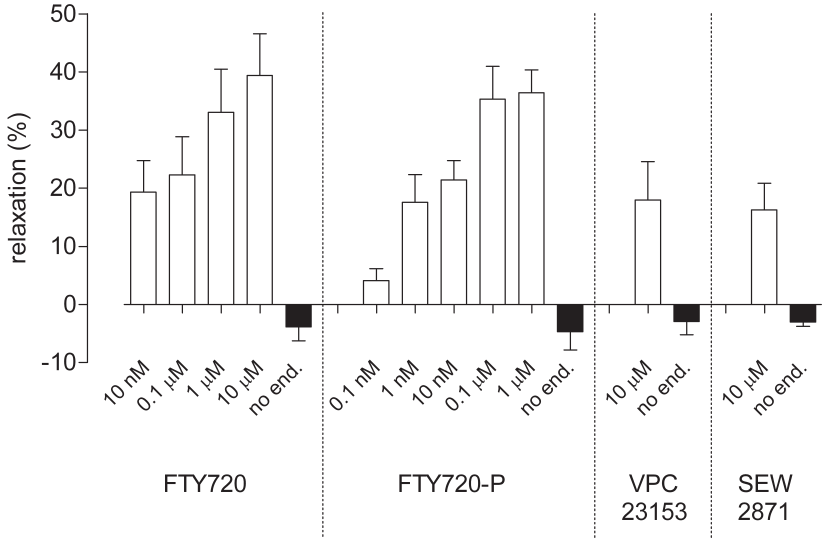


Figure 2. Ligand-induced relaxation of preconstricted (1 µM phenylephrine) rat aortic rings with (white bars) and without (black bars) endothelium by FTY720, FTY720-P, VPC23153 and SEW2871. Data are shown as % relaxation of phenylephrine-induced contraction and are means ± s.e.m. of 2-5 experiments.

tion, but with a lower potency (pEC_{50} = 6.2 ± 0.9 (n=5) and 5.9 ± 0.2 (n=8), respectively) than the two former compounds (figure 3A). Surprisingly, the $S1P_{1,3}$ agonist VPC23153 (Table 1, Figure 1) did not decrease but rather increased forskolin-induced cAMP accumulation (pEC_{50} = 5.8 ± 0.3 (n=3)) (Figure 3B). The $S1P_{1,3}$ antagonist VPC23019 (Table 1, Figure 1) concentration-dependently shifted the FTY720-P curve to the right yielding a Schild plot with a slope close to unity and a pK_B of 7.4 (Figure 4). The $S1P_2$ receptor antagonist JTE-013 (Table 1, Figure 1)

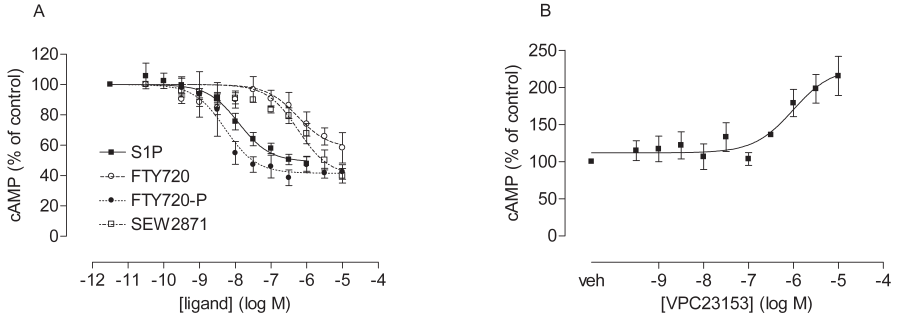


Figure 3. S1P, FTY720 and FTY720-P and SEW2871-induced inhibition of forskolin (3 µM)-induced cAMP accumulation in RAECs (A). Data are expressed as % of forskolin-induced cAMP production of vehicle control (100% = 16.6 ± 3.7 nM (S1P, n=10), 19.0 ± 4.4 nM (SEW2871, n=8), 19.0 ± 6.9 nM (FTY720, n=5), 16.9 ± 5.9 nM (FTY720-P, n=5). Effect of VPC23153 on forskolin (3 µM)-induced cAMP accumulation in RAECs (B). (100% = 6.4 ± 2.1 nM (n=3)).

(10 µM) did not affect concentration response curves of agonist-mediated inhibition of forskolin-induced cAMP levels (data not shown). Both S1P and FTY720-P slightly increased $[Ca^{2+}]_i$ in RAECs but only at high concentrations (1 µM) (107 ±17 nM and 96 ± 43 nM, respectively), whereas the other ligands failed to do so.

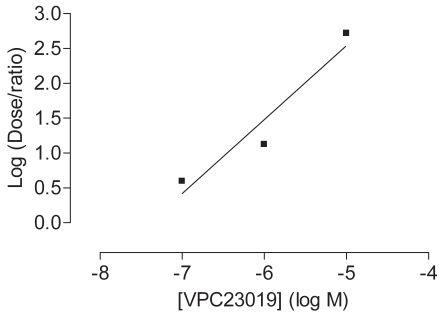


Figure 4. Schild plot of the effect of VPC23019 on the FTY720-P-induced inhibition of forskolin-induced cAMP accumulation in RAECs. Cells were pre-incubated for 30 min with the indicated concentrations of VPC23019. (n=6). Slope = 1.06, pK_B =7.4.

S1P receptor-mediated signaling in VSMCs

In contrast to the endothelial cells, S1P, FTY720-P and SEW2871 did not affect forskolin-induced cAMP accumulation in rat aortic VSMCs to an extent that was reliably quantifiable. However, S1P induced an elevation of $[Ca^{2+}]_i$ in VSMCs, which was inhibited by JTE-013 (Figure 5) ($p<0.05$). This antagonist did not significantly inhibit ATP-induced increases in $[Ca^{2+}]_i$ (vehicle: pEC_{50} = 5.5 ± 0.1, E_{max} = 1539 ± 402 nM (n=5); JTE-013 (1 µM): pEC_{50} = 5.4 ± 0.2, E_{max} = 1404 ± 403 nM (n=4)). VPC23019, VPC23153, FTY720 and FTY720-P did not elevate $[Ca^{2+}]_i$.

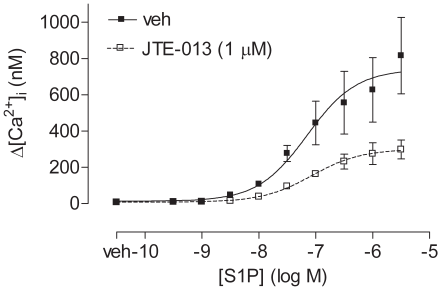


Figure 5. The effect of JTE-013 (1 µM) on concentration-dependent S1P-induced elevation of $[Ca^{2+}]_i$ in VSMCs. E_{max} = 816 ± 211 nM (veh), 298 ± 52* nM (1 µM) and 180 ± 40* nM (10 µM). * $p<0.05$ (n=5).

S1P receptor mRNA expression

Quantitative PCR showed the presence of $S1P_1$, $S1P_2$ and $S1P_3$ receptor mRNA in VSMCs (C_t value ranging from 30-32 (n=3), corrected for P0 and EF-1). RAECs only showed $S1P_1$ receptor mRNA expression (approximately 46 times higher than in VSMCs) (C_t value around 25 (n=3), corrected for P0 and EF-1), whereas mRNA for the $S1P_2$ and $S1P_3$ receptors was not detected up to cycle 40.

Discussion

Besides regulators of growth, sphingolipids (including S1P) are vasoactive compounds. S1P has been shown to induce vasodilation and vasoconstriction, amongst others depending on vessel type, animal species and technique used (reviewed in Hemmings, 2006). These processes are most likely mediated by high affinity S1P receptors, although the existence of intracellular targets of S1P cannot be ruled out (Payne et al., 2002). Within the vasculature, mainly the $S1P_{1-3}$ receptor subtypes are expressed and the ultimate effect of S1P on a given vessel is, therefore, dependent on the differential expression of these receptor subtypes in the endothelium and smooth muscle cells. This study was performed to use agonists and antagonists of the S1P receptors that have become recently available to pharmacologically characterize S1P receptor-dependent signaling in intact isolated rat aortic rings and cultured rat aortic endothelial and vascular smooth muscle cells. The immunosuppressant drug candidate FTY720 (fingolimod, now being developed for the treatment of multiple sclerosis (Kappos et al., 2006)) can be phosphorylated by sphingosine kinase and in its phosphorylated form (FTY720-P) is a potent agonist for all, except the $S1P_2$ receptor subtype (Mandala et al., 2002). VPC23153 is described to be an agonist at the human $S1P_1$ and $S1P_3$ receptor, whereas VPC23019 has been reported to have antagonistic effects at these receptors. SEW2871 is a highly selective $S1P_1$ agonist and JTE013 displays antagonistic activity towards the $S1P_2$ receptor (Table 1, Figure 1).

Both endothelial and vascular smooth muscle cells express S1P receptors (Michel et al., 2007) and the intracellular signaling pathways these receptors activate are known to affect vascular tone (Alewijnse et al., 2004; Young & Van Brocklyn, 2006; Hemmings, 2006). Indeed, all of the aforementioned compounds tested in this study, with the exception of S1P, modulated vascular tone in rat aortic rings as they induced a concentration-dependent vasorelaxation in pre-constricted preparations (Figure 1). Under non-preconstricted conditions all compounds were devoid of any

activity. The observed relaxation was abolished by endothelium removal.
qPCR analysis in cultured RAECs showed high mRNA expression of the G_i-coupled $S1P_1$ receptor, whereas $S1P_2$ and $S1P_3$ receptor mRNA was not detected. Intracellular calcium levels in RAECs were only slightly increased by high concentrations (1 μM) of S1P or FTY720-P (less than 20% compared to VSMC responses), which might be due to non-specific effects.
With the exception of VPC23153, all tested agonists (FTY720, FTY720-P, S1P and SEW2871) inhibited forskolin-induced cAMP accumulation in RAECs. The order of potency of these compounds in this assay matched with their $S1P_1$ receptor potency (Table 1, Figure 1). VPC23153 did not decrease, but rather increased the forskolin-induced cAMP accumulation, indicating the possibility of inverse agonism. Both, increase and decrease in cAMP production in ECs were associated with vasorelaxation, indicating that this effect is not crucial for the final vasorelaxant effect. Therefore, the endothelium-dependent vasorelaxant effect seen in aorta preparations is most likely mediated via βγ-subunits of G_i, as was recently shown (Gonzalez et al., 2006).

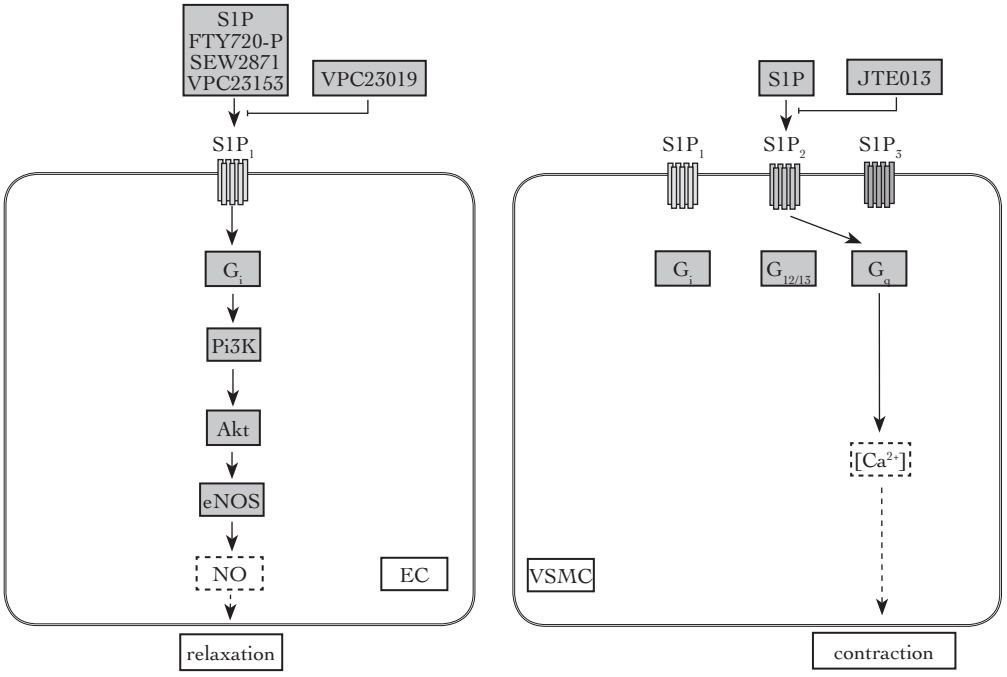


Figure 6. Schematic representation of the effect of S1P receptor ligands in ECs and VSMCs. $S1P_1$, $S1P_2$ and $S1P_3$ receptors activate signaling pathways leading to contraction or relaxation. Activation of the $S1P_1$ and probably also $S1P_3$ receptors in ECs lead to relaxation. In VSMCs, activation of $S1P_2$ receptors leads to contraction.

qPCR showed mRNA expression for the $S1P_1$, $S1P_2$ and $S1P_3$ receptors in cultured VSMCs. In line with these findings, FTY720, FTY720-P and SEW2871 inhibited forskolin-induced cAMP accumulation in these cells. However, this effect was relatively small, making reliable quantification not possible. Increases in $[Ca^{2+}]_i$ were only observed after stimulation with S1P, but not with any of the other ligands, which all lack $S1P_2$ agonism. Additionally, JTE-013 inhibited this S1P-induced elevation of $[Ca^{2+}]_i$. These findings indicate that in smooth muscle cells, S1P effects are predominantly mediated via the $S1P_2$ receptor.

An overview of the effects of S1P and the new synthetic S1P receptor ligands on either EC and/or VSMC is drawn in figure 6. Our results show that in intact aortic rings, endothelium-dependent relaxation is induced by all synthetic agonists, most likely via the activation of the $S1P_1$ receptor on endothelial cells. However, the presence and influence of the $S1P_3$ receptor in these cells can not be ruled out. On VSMCs, only the ligand with $S1P_2$ agonistic activity, being S1P itself, increased $[Ca^{2+}]_i$, indicating that S1P signaling in these cells occurs predominantly via the $S1P_2$ receptor. Specific $S1P_2$ receptor activation in aortic rings can however not be investigated as these compounds do not act on $S1P_2$ receptors. Therefore, the net effect of these new ligands in the aorta shall be relaxation. In disease states that are characterized by vascular dysfunction, e.g. hypertension, diabetes or atherosclerosis, subtype selective S1P receptor agonist/antagonists may be used as modulators of vascular tone.

References

Albert R, Hinterding K, Brinkmann V, Guerini D, Muller-Hartwieg C, Knecht H, Simeon C, Streiff M, Wagner T, Welzenbach K, Zecri F, Zollinger M, Cooke N, Francotte E (2005). Novel immunomodulator FTY720 is phosphorylated in rats and humans to form a single stereoisomer. Identification, chemical proof, and biological characterization of the biologically active species and its enantiomer. *J Med Chem* **48**:5373-5377

Alewijnse AE, Peters SL, Michel MC (2004). Cardiovascular effects of sphingosine-1-phosphate and other sphingomyelin metabolites. *Br J Pharmacol* **143**:666-684

Davis MD, Clemens JJ, Macdonald TL, Lynch KR (2005). Sphingosine 1-phosphate analogs as receptor antagonists. *J Biol Chem* **280**:9833-9841

Gonzalez E, Kou R, Michel T (2006). Rac1 modulates sphingosine 1-phosphate-mediated activation of phosphoinositide 3-kinase/Akt signaling pathways in vascular endothelial cells. *J Biol Chem* **281**:3210-3216

Hannun YA, Luberto C, Argraves KM (2001). Enzymes of sphingolipid metabolism: from modular to integrative signaling. *Biochemistry* **40**:4893-4903

Hemmings DG (2006). Signal transduction underlying the vascular effects of sphingosine 1-phosphate and sphingosylphosphorylcholine. *Naunyn Schmiedebergs Arch Pharmacol* **373**:18-29

Kappos L, Antel J, Comi G, Montalban X, O'Connor P, Polman CH, Haas T, Korn AA, Karlsson G, Radue EW (2006) Oral fingolimod (FTY720) for relapsing multiple sclerosis. *N Engl J Med* **355**:1124-1140

Kluk MJ, Hla T (2001). Role of the sphingosine 1-phosphate receptor EDG-1 in vascular smooth muscle cell proliferation and migration. *Circ Res* **89**:496-502

Mandala S, Hajdu R, Bergstrom J, Quackenbush E, Xie J, Milligan J, Thornton R, Shei GJ, Card D, Keohane C, Rosenbach M, Hale J, Lynch CL, Rupprecht K, Parsons W, Rosen H (2002). Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. *Science* **296**:346-349

Michel MC, Mulders AC, Jongsma M, Alewijnse AE, Peters SL (2007). Vascular effects of sphingolipids. *Acta Paediatr Suppl* **96**:44-48

Morales-Ruiz M, Lee MJ, Zöllner S, Gratton JP, Scotland R, Shiojima I, Walsh K, Hla T, Sessa WC (2001). Sphingosine 1-phosphate activates Akt, nitric oxide production, and chemotaxis through a Gi protein/phosphoinositide 3-kinase pathway in endothelial cells. *J Biol Chem* **276**:19672-19677

Mulders AC, Hendriks-Balk MC, Mathy MJ, Michel MC, Alewijnse AE, Peters SL (2006). Sphingosine kinase-dependent activation of endothelial nitric oxide synthase by angiotensin II. *Arterioscler Thromb Vasc Biol* **26**:2043-2048

Murata N, Sato K, Kon J, Tomura H, Yanagita M, Kuwabara A, Ui M, Okajima F (2000). Interaction of sphingosine 1-phosphate with plasma components, including lipoproteins, regulates the lipid receptor-mediated actions. *Biochem J* **352**:809-815

Nofer JR, van der Giet M, Tolle M, Wolinska I, von Wnuck Lipinski K, Baba HA, Tietge UJ, Godecke A, Ishii I, Kleuser B, Schafers M, Fobker M, Zidek W, Assmann G, Chun J, Levkau B (2004). HDL induces NO-dependent vasorelaxation via the lysophospholipid receptor S1P3. *J Clin Invest* **113**:569-581

Ohmori T, Yatomi Y, Osada M, Kazama F, Takafuta T, Ikeda H, Ozaki Y (2003). Sphingosine 1-phosphate induces contraction of coronary artery smooth muscle cells via S1P2. *Cardiovasc Res* **58**:170-177

Pappu R, Schwab SR, Cornelissen I, Pereira JP, Regard JB, Xu Y, Camerer E, Zheng YW, Huang Y, Cyster JG, Coughlin SR (2007). Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. *Science* **316**:295-298

Payne SG, Milstien S, Spiegel S (2002). Sphingosine-1-phosphate: dual messenger functions. FEBS Lett 531:54-57

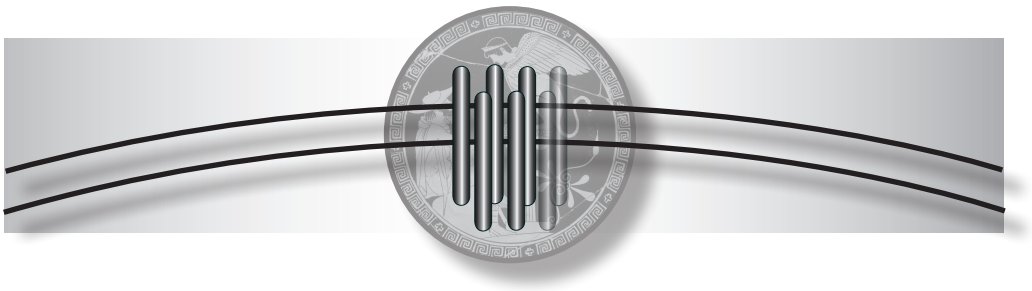
Peters SL, Alewijnse AE (2007). Sphingosine-1-phosphate signaling in the cardiovascular system. *Curr Opin Pharmacol* **7**:186-192

Sanna MG, Liao J, Jo E, Alfonso C, Ahn MY, Peterson MS, Webb B, Lefebvre S, Chun J, Gray N, Rosen H (2004). Sphingosine 1-phosphate (S1P) receptor subtypes S1P1 and S1P3, respectively, regulate lymphocyte recirculation and heart rate. *J Biol Chem* **279**:13839-13848

Waeber C, Blondeau N, Salomone S (2004). Vascular sphingosine-1-phosphate S1P1 and S1P3 receptors. *Drug News Perspect* **17**:365-382

Yatomi Y, Ohmori T, Rile G, Kazama F, Okamoto H, Sano T, Satoh K, Kume S, Tigyi G, Igarashi Y, Ozaki Y (2000). Sphingosine 1-phosphate as a major bioactive lysophospholipid that is released from platelets and interacts with endothelial cells. *Blood* **96**:3431-3438

Young N, Van Brocklyn JR (2006). Signal transduction of sphingosine-1-phosphate G protein-coupled receptors. *ScientificWorldJournal* **6**:946-966



Differential agonist-induced internalization of sphingosine-1-phosphate receptor subtypes 1, 2 and 3

Chapter 5

Differential agonist-induced internalization of sphingosine-1-phosphate receptor subtypes 1, 2 and 3

Manuscript in preparation

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Abstract

Regulation of sphingosine-1-phosphate (S1P) receptors is of major importance especially since it has been shown to be the mechanism of action for the immunosuppressive prodrug FTY720. Little is known about possible differential regulation of the different S1P receptor subtypes expressed in the cardiovascular system. This study was therefore set up to compare ligand-induced internalization of the $S1P_{1-3}$ receptor subtypes. A recently developed immunocytochemical method was used to characterize internalization and resensitization of membrane expressed HisG-tagged S1P receptors. Inhibitors of several downstream signaling pathways were used to investigate the mechanism of receptor internalization. $S1P_3$ receptor internalization showed striking differences compared to $S1P_1$ and $S1P_2$ receptor internalization as it occurred much faster, required lower S1P concentrations and showed a higher reduction in the fraction of membrane receptors. The differences in signaling between the three receptor subtypes could only partly explain the differential internalization of these receptors. For the $S1P_3$ receptor activation of G_i-proteins is involved in $S1P_3$ receptor internalization, whereas elevations in $[Ca^{2+}]_i$ or activation of G_q-signaling are not important.

In conclusion, the S1P receptor subtypes expressed in the cardiovascular system differ in ligand-induced internalization, with G_i-activation being of influence. This difference could be important for the functioning of drugs targeted at S1P receptors, including FTY720.

Introduction

The bioactive lipid sphingosine-1-phosphate (S1P) is involved in the regulation of many important cellular processes such as migration and proliferation and lymphocyte trafficking. Most of the effects of S1P are mediated via five distinct G-protein coupled receptors (GPCRs), named $S1P_{1-5}$ (previously EDG-1,5,3,6 and 8, respectively) (Chun et al., 2002). The $S1P_1$, $S1P_2$ and $S1P_3$ receptors are ubiquitously expressed and are the most important sphingolipid receptors in the cardiovascular system (Spiegel & Milstien, 2000; Alewijnse et al., 2004), the $S1P_4$ receptor is mainly expressed in the lung and lymphoid tissues and the $S1P_5$ receptor in the white matter of the central nervous system (Anliker & Chun, 2004). S1P can be generated in many cell types by the concerted action of specific enzymes and its synthesis is tightly controlled through the regulation of activity of specific enzymes such as sphingosine kinase (Spiegel & Milstien, 2003).

In general, exposure of GPCRs to agonists initiates a series of regulatory processes, some of which directly affect receptor responsiveness. One of these mechanisms involves arrestin binding upon phosphorylation of the receptor by G-protein coupled receptor kinases. This uncouples the receptor from the G-protein and results in termination of signaling. Another mechanism is internalization of the receptor, either arrestin-dependent or independent (Bünemann & Hosey, 1999), and involves translocation of the receptor from the plasma membrane to clathrin-coated pits inside the cell from where it can either be recycled to the membrane or undergo degradation. It is nowadays well accepted that GPCR internalization not only regulates the functional activity and number of receptors present at the cell surface, but that it also mediates GPCR coupling to certain downstream effector pathways (von Zastrow, 2001).

As found for other GPCRs, S1P receptors are also subject to receptor regulatory processes upon ligand stimulation. High concentrations of S1P, 200-900 nM (Okajima, 2002), have been found in plasma and serum, suggesting that S1P is present in blood. The origin of S1P in blood, recently studied in mouse, was found to be mainly haematopoietic with erythrocytes being the most important contributor (Pappu et al., 2007). The concentrations of S1P in blood are sufficient to stimulate S1P receptors on cardiovascular cells such as endothelial cells and probably also smooth muscle cells, but it is not known whether this actually occurs, since the majority of S1P in blood in bound to lipoproteins, stored in platelets or bound to albumin (Okajima, 2002). Although it is still unclear if bound S1P is indeed func-

tionally active, regulatory processes can be speculated to be important in the cardiovascular system. Interestingly, S1P receptor internalization is suggested to be the mechanism of action of FTY720, an immunosuppressive prodrug that is now in clinical trials for the treatment of multiple sclerosis (Kappos et al., 2006). Although FTY720, once phosphorylated in vivo by sphingosine kinase-2, is a potent agonist for four of the five S1P receptors ($S1P_{1,3-5}$) (Mandala et al., 2002), the lymphopenia induced by this drug is suggested to be mainly due to internalization of the $S1P_1$ receptor expressed on lymphocytes (Matloubian et al., 2004). Besides the importance of $S1P_1$ receptor regulation in the immune system, this process may also be of major importance for the S1P receptors in the cardiovascular system. Except from some recent work on the $S1P_1$ and $S1P_3$ receptor (Liu et al., 1999; Spiegel & Milstien, 2000; Rutherford et al., 2005), relatively little is known about regulation of the S1P receptors. Therefore, we have investigated differences in regulation upon agonist treatment of the S1P receptor subtypes important in the cardiovascular system. For this purpose, we compared internalization of the $S1P_1$, $S1P_2$ and $S1P_3$ receptors using a recently described method that enables quantification of receptor internalization (Chapter 3). Our research revealed that these three S1P receptor subtypes differ strikingly in ligand-induced internalization and that these differences can partly be explained by differences in signaling properties between these receptor subtypes. For the $S1P_3$ receptor the internalization is partly dependent on G_i-activation whereas the activation of G_q-signaling pathways was not important.

Materials & Methods

Materials

pcDNA3.1 containing the entire coding region of human $S1P_{1-3}$ receptors was purchased from UMR cDNA Resource Center (Rolla, MO, USA). Cell culture media, hygromycine B, Lipofectamine™2000, pOG44, CHO-FlpIn cells, Alexa Fluor® 488 goat anti-mouse (IgG), anti-HisG (mouse monoclonal IgG2a), anti-HisG-horse radish peroxidase (HRP) (mouse monoclonal IgG2a) and pcDNA5/FRT/TO were obtained from Invitrogen (Breda, The Netherlands). Restriction enzymes (ApaI, BamHI, XhoI, EcoRI and HindIII) were obtained from Fermentas Life Sciences (St. Leon-Rot, Germany). Black, clear bottom 96 well plates were obtained from Greiner Bio One (Alphen aan den Rijn, The Netherlands). S1P was obtained from Avanti-Polar Lipids, Inc (via Instruchemie B.V., Delfzijl, the Netherlands). Pertussis toxin, A23187, BAPTA-AM (1,2-Bis (2-aminophenoxy)ethane-N,N,N',N'-

tetraacetic acid, tetraacetoxymethyl ester), calphostine-C, U73122 (1-[6-[((17ß)-3-Methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione) and Rp-cAMPS (Rp-Cyclic 3',5'-hydrogen phosphorothioate adenosine triethylammonium salt) were obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands).

Molecular cloning

A N-terminal HisG-tag was added to the $S1P_{1-3}$ receptors via cloning into pcDNA3.1/HisC using BamHI&XhoI (for $S1P_1$) or BamHI&ApaI (for $S1P_2$) or into pcDNA3.1/HisA using BamHI&XhoI (for $S1P_3$). A second cloning step was done using HindIII&XhoI (for $S1P_{1,3}$) or HindIII&ApaI (for $S1P_2$) to clone the HisG-tagged S1P receptors into the expression vector pcDNA5/FRT/TO. The cDNA of the $S1P_3$ receptor obtained from Guthrie contained a point mutation ($G^{962}A$ which results in $R^{321}Q$) which was removed by PCR using the following primers (forward: TGCCTGGTCAGGGGGCGGGGGGCCCG, reverse: CGGGCCCCCCGCCCCCTGACCAGGCA). The $S1P_{1-3}$ receptor coding DNA in the resulting plasmid as well as the stable cell line constructed with this plasmid have been confirmed by sequencing.

Transfection and cell culture

Generation of stable CHO-FlpIn cell lines expressing the indicated receptors and culturing of these cell lines was done as described previously (Chapter 2). The cell lines were checked for expression of the indicated HisG-S1P receptors by immunocytochemistry as described below. These experiments revealed no major differences in membrane fluorescence for the three receptor subtypes.

Calcium assay and cAMP assay

Intracellular calcium concentrations ($[Ca^{2+}]_i$) and cAMP levels were measured as described previously (Chapter 2).

Immunocytochemistry

Immunocytochemistry was performed as described previously (Chapter 3). In short, an N-terminal HisG-tag was added to the receptors which can be detected using a 1st anti-HisG antibody combined with a AlexaFluor® 488 goat-anti-mouse fluorescent 2nd antibody. The fluorescent signal can be measured on a microplate reader. Some adaptations were made for specific applications of this method. After overnight serum starvation, cells were stimulated with the indicated ligand in

serum free medium for 30 minutes at 37 °C (for concentration response curves), stimulated with 1 µM S1P for the indicated time (for time course stimulations) or stimulated with 1 µM S1P for 30 minutes at 37 °C followed by 3 times washing with serum free medium and incubation at 37 °C with serum free medium for the indicated time (for resensitization).

Data analysis

Concentration response curves were analyzed by fitting sigmoidal functions to the experimental data using Prism 4 (Graphpad Software, Inc., San Diego, CA, USA). Data are expressed as means ± s.e.m. One-way ANOVA with a Dunnett's correction or Student's t-test was applied where appropriate. $P<0.05$ was considered significant.

Results

Time course of internalization of the HisG-S1P$_1$, HisG-S1P$_2$ and HisG-S1P$_3$ receptor

Internalization of the different receptor subtypes was studied by determining the decrease of membrane receptors by an immunocytochemical method. For all receptors studied, incubation with 1 µM S1P induced a time-dependent decrease in cell surface receptors. Internalization of the HisG-S1P$_3$ receptor was significantly faster ($t_{½}$ =1.1 ± 0.2 min (n=4)) than of the HisG-S1P$_1$ ($t_{½}$ = 16.6 ± 7.0 min (n=4)) and HisG-S1P$_2$ receptors ($t_{½}$ = 19.1 ± 5.4 min (n=4)) ($p<0.05$) (Figure 1). All further internalization experiments were done with 30 min agonist exposure.

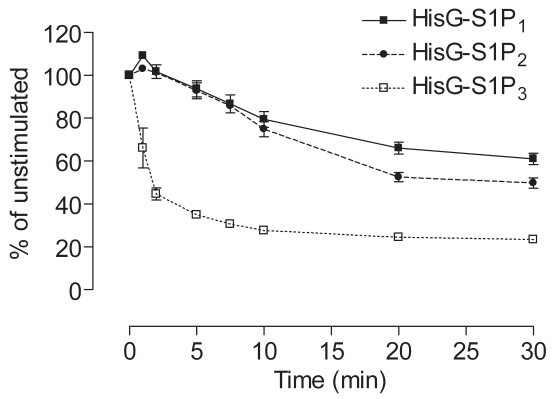


Figure 1. Time dependent S1P-induced internalization of HisG-S1P$_1$, HisG-S1P$_2$ or HisG-S1P$_3$ receptor subtypes. Stimulations with S1P (1 µM) were carried out at 37 ºC for the indicated time. Values are expressed as percentage of fluorescent signal measured for unstimulated cells (t = 0 min) which was set at 100% and are presented as means ± s.e.m. (100% was for HisG-S1P$_1$: 54978 ± 5892 RFU (n=5); HisG-S1P$_2$: 65091 ± 4610 RFU (n=5); HisG-S1P$_3$: 62013 ± 4941 RFU (n=5)).

Potency of internalization of the HisG-S1P$_1$, HisG-S1P$_2$ and HisG-S1P$_3$ receptor

The potency of S1P to induce internalization was similar for the HisG-S1P$_1$ and HisG-S1P$_2$ receptor (pEC$_{50}$ = 7.5 ± 0.2 (n=8) and 7.1 ± 0.2 (n=4)), while the HisG-S1P$_3$ receptor was more sensitive to S1P-induced internalization (pEC$_{50}$ = 8.0 ± 0.2 (n=3)) (Figure 2). Besides a difference in pEC$_{50}$ between the subtypes in response to S1P, also the relative amount of internalization differed. Thus, the HisG-S1P$_3$ receptor internalized to a lower level than the HisG-S1P$_1$ or the HisG-S1P$_2$ receptor (HisG-S1P$_3$: 27 ± 3 % (n=3) vs HisG-S1P$_1$: 58 ± 2 % (n=8) and HisG-S1P$_2$: 42 ± 2 % (n=4), $p<0.05$).

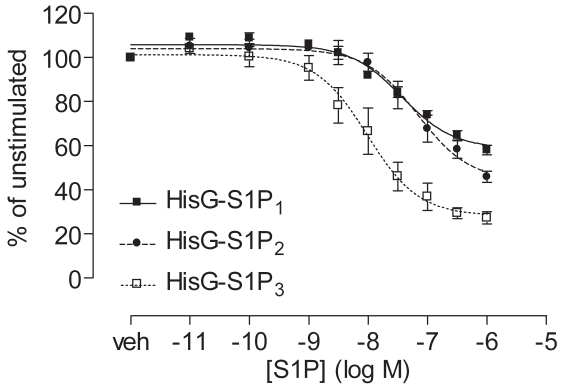


Figure 2. Concentration-dependent S1P-induced internalization of HisG-S1P$_1$, HisG-S1P$_2$ or HisG-S1P$_3$ receptor subtypes. Stimulations were carried out at 37 ºC for 30 min. Values are expressed as percentage of fluorescent signal measured for unstimulated cells which was set at 100% and are presented as means ± s.e.m. (100% was for HisG-S1P$_1$: 49374 ± 3620 RFU (n=8); HisG-S1P$_2$: 63161 ± 3235 RFU (n=4); HisG-S1P$_3$: 53837 ± 5899 RFU (n=3)).

Resensitization

Recycling of S1P receptors to the membrane was studied. The washing procedure that followed initial S1P or vehicle stimulation reduced the amount of membrane receptors for the HisG-S1P$_3$ receptor, but not for the HisG-S1P$_1$ or HisG-S1P$_2$ receptor. Therefore, we quantified the difference in membrane fluorescence between 30 min vehicle-treated and S1P-stimulated cells at different times after the initial stimulation. Significant differences remained in this regard for 1.5 h after washout of S1P for the HisG-S1P$_2$ receptor (Figure 3B) and for 2 h after washout of S1P for the HisG-S1P$_1$ receptor (Figure 3A). For the HisG-S1P$_3$ receptor the difference between the vehicle and S1P treated cells remained significant for at least 4 h after washout of S1P, but the fluorescence level was only restored to 80 % (yet still increasing) of the initial level (Figure 3C).

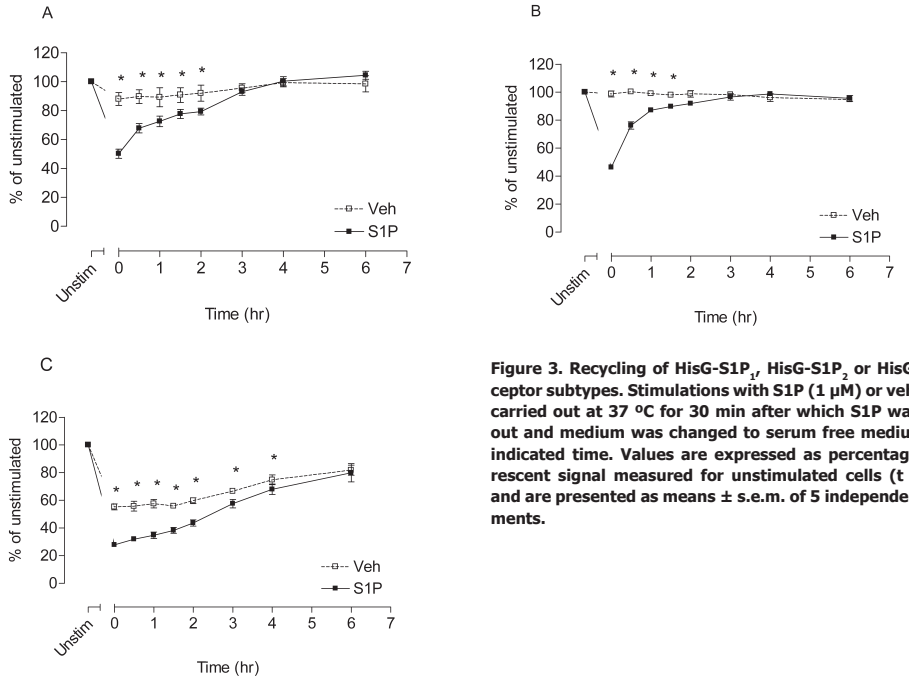


Figure 3. Recycling of HisG-S1P$_1$, HisG-S1P$_2$ or HisG-S1P$_3$ receptor subtypes. Stimulations with S1P (1 µM) or vehicle were carried out at 37 ºC for 30 min after which S1P was washed out and medium was changed to serum free medium for the indicated time. Values are expressed as percentage of fluorescent signal measured for unstimulated cells (t = 0 min) and are presented as means ± s.e.m. of 5 independent experiments.

Signaling of the HisG-S1P$_1$, HisG-S1P$_2$ and HisG-S1P$_3$ receptor

Previously it was shown that the HisG tag did not affect signaling of the S1P$_1$ receptor as no difference in the potency of S1P to inhibit forskolin-induced cAMP accumulation in the cells expressing the tagged or untagged S1P$_1$ receptor was seen (Chapter 3). Also, no difference was observed after addition of the HisG tag on signaling of the S1P$_2$ and S1P$_3$ receptor (data not shown). There was no change in the

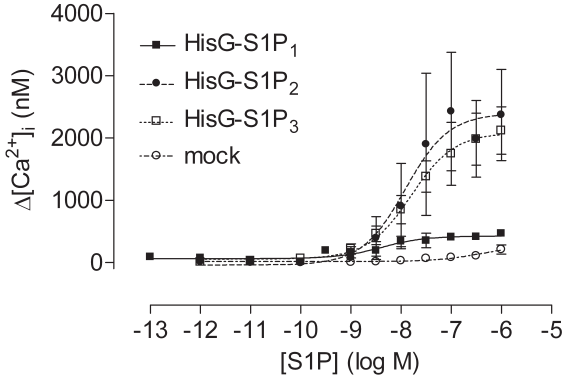


Figure 4. S1P-induced increases in $[Ca^{2+}]_i$ in mock transfected CHO-FlpIn or in CHO-FlpIn cells expressing the HisG-S1P$_1$, HisG-S1P$_2$ or HisG-S1P$_3$ receptor or in mock transfected cells. Data presented are means ± s.e.m. of 3 to 6 independent experiments.

potency of S1P to inhibit forskolin-induced cAMP accumulation in cells expressing the $S1P_3$ receptor and the HisG tag did not influence the potency of S1P to raise intracellular calcium levels in cells expressing the $S1P_2$ receptor (data not shown). S1P slightly but significantly increased elevations in $[Ca^{2+}]_i$ in cells expressing the HisG-$S1P_1$ receptor compared to mock transfected cells (HisG-$S1P_1$: E_{max} = 499 ± 29 nM (n=3) and mock: E_{max} = 205 ± 74 nM (n=3)). Cells expressing the HisG-$S1P_2$ receptor or the HisG-$S1P_3$ receptor showed significantly higher elevations in $[Ca^{2+}]_i$ upon S1P stimulation compared to mock transfected cells and cells expressing the HisG-$S1P_1$ receptor (HisG-$S1P_2$: pEC_{50} = 7.6 ± 0.3; E_{max} = 2614 ± 813 nM (n=4) and HisG-$S1P_3$: pEC_{50} = 7.9 ± 0.3; E_{max} = 2166 ± 438 nM (n=6)) (Figure 4).

S1P did not inhibit the forskolin-induced cAMP accumulation in mock transfected cells (data not shown) or in cells transfected with the HisG-$S1P_2$ receptor (Figure 5). However, S1P inhibited forskolin-induced cAMP accumulation in cells expressing the HisG-$S1P_1$ (pEC_{50} = 9.5 ± 0.2; E_{max} = 22 ± 4 % (n=11)) or HisG-$S1P_3$ receptor (pEC_{50} = 8.6 ± 0.2; E_{max} = 21 ± 1 % (n=9)) (Figure 5). Surprisingly, S1P increased the forskolin-induced cAMP accumulation via the HisG-$S1P_2$ receptor (pEC_{50} = 7.0 ± 0.2; E_{max} = 170 ±11 % (n=3)).

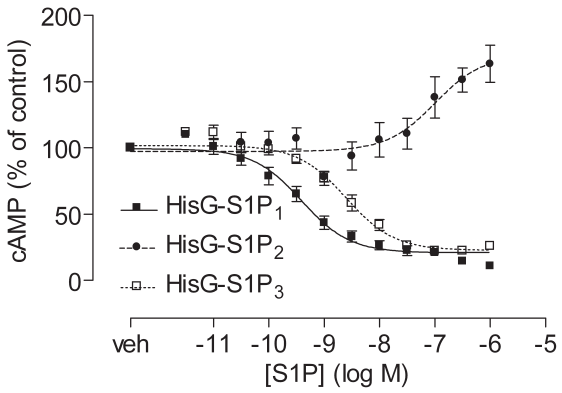


Figure 5. S1P-mediated effect on forskolin-induced cAMP accumulation in cells expressing the HisG-$S1P_1$, HisG-$S1P_2$ or HisG-$S1P_3$ receptor. Stimulations were carried out for 60 min at room temperature. The forskolin-induced cAMP accumulation in the absence of S1P was set at 100% and all values were expressed compared to this control (100 % was for HisG-$S1P_1$: 14.4 ± 1.9 nM (n=11); HisG-$S1P_2$: 4.3 ± 0.7 nM (n=3); HisG-$S1P_3$: 15.7 ± 1.3 nM (n=9)).

Influence of pertussis toxin (PTX) on internalization of the HisG-S1P$_1$, HisG-S1P$_2$ and HisG-S1P$_3$ receptor

The S1P-induced internalization of the HisG-S1P$_1$ and HisG-S1P$_2$ receptor were not significantly altered by overnight incubations with PTX (100 ng/ml (S1P-induced inhibition of cAMP accumulation was completely abolished under these conditions)) (pEC$_{50}$ = 7.2 ± 0.1 vs 6.9 ± 0.1 (n=4) for HisG-S1P$_1$; 6.8 ± 0.1 vs 6.8 ± 0.1 for HisG-S1P$_2$ (n=3)) (Figure 6 A,B). However, PTX slightly but significantly attenuated S1P-induced internalization of the HisG-S1P$_3$ receptor (pEC$_{50}$ = 8.1 ± 0.1 (without PTX) and 7.3 ± 0.1 (with PTX) (n=4)) (Figure 6C).

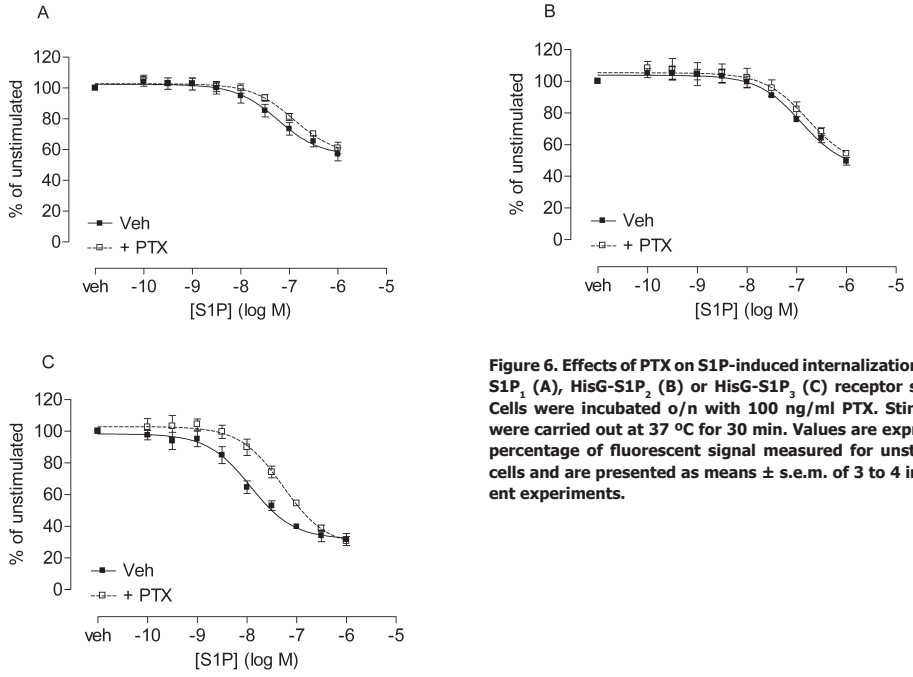


Figure 6. Effects of PTX on S1P-induced internalization of HisG-S1P$_1$ (A), HisG-S1P$_2$ (B) or HisG-S1P$_3$ (C) receptor subtypes. Cells were incubated o/n with 100 ng/ml PTX. Stimulations were carried out at 37 ºC for 30 min. Values are expressed as percentage of fluorescent signal measured for unstimulated cells and are presented as means ± s.e.m. of 3 to 4 independent experiments.

Signal transduction involved in internalization of HisG-S1P$_1$, HisG-S1P$_2$ and HisG-S1P$_3$ receptors

Pre-incubations with either the protein kinase A (PKA) inhibitor Rp-cAMPS (100 µM), the phospholipase C (PLC) inhibitor U73122 (10 µM) or the protein kinase C (PKC) inhibitor calphostine-C (1 µM) did not alter S1P-induced HisG-S1P$_3$ receptor internalization (veh: pEC$_{50}$ = 7.8 ± 0.2 (n=5); Rp-cAMPs: pEC$_{50}$ = 7.9 ± 0.2 (n=4); U73122: pEC$_{50}$ = 8.0 ± 0.1 (n=3); calphostine-C: pEC$_{50}$ = 8.1 ± 0.4 (n=3)) (Table 1). The S1P-induced internalization of the other two subtypes was also not significantly altered by the different inhibitors (Table 1).

Table 1. Influence of Rp-cAMPS (100 µM), calphostin-C (1 µM) and U73122 (10 µM) on S1P-induced internalization of the HisG-S1P$_1$, HisG-S1P$_2$ or HisG-S1P$_3$ receptor subtypes. Stimulations were carried out at 37 ºC for 30 min. Values are expressed as percentage of fluorescent signal measured for unstimulated cells and are presented as means ± s.e.m.

	veh	Rp-cAMPs	U73122	calphostine-C
HisG-S1P$_1$	7.1 ± 0.1 (5)	7.0 ± 0.2 (4)	7.0 ± 0.1 (2)	7.2 ± 0.2 (3)
HisG-S1P$_2$	6.8 ± 0.0 (5)	6.8 ± 0.1 (4)	6.5 ± 0.2 (3)	6.7 ± 0.2 (3)
HisG-S1P$_3$	7.8 ± 0.2 (5)	7.9 ± 0.2 (4)	8.0 ± 0.1 (3)	8.1 ± 0.4 (3)

Addition of the calcium ionophore A23187 (10 µM) did not influence HisG-S1P$_1$ receptor internalization (pEC$_{50}$ = 7.0 ± 0.1 vs 7.0 ± 0.2 (n=3) (vehicle vs A23187)) (Figure 7A). Addition of the calcium chelator BAPTA-AM (10 µM) also had no significant influence on HisG-S1P$_3$ receptor internalization (pEC$_{50}$ = 7.6 ± 0.2 vs 8.2 ± 0.2. (n=3) (vehicle vs BAPTA-AM)) (Figure 7B). This BAPTA-AM concentration fully suppressed increases in $[Ca^{2+}]_i$ in CHO cells expressing the HisG-S1P$_3$ receptor, while the used A23187 concentration induced a similar increase in $[Ca^{2+}]_i$ compared to S1P (data not shown).

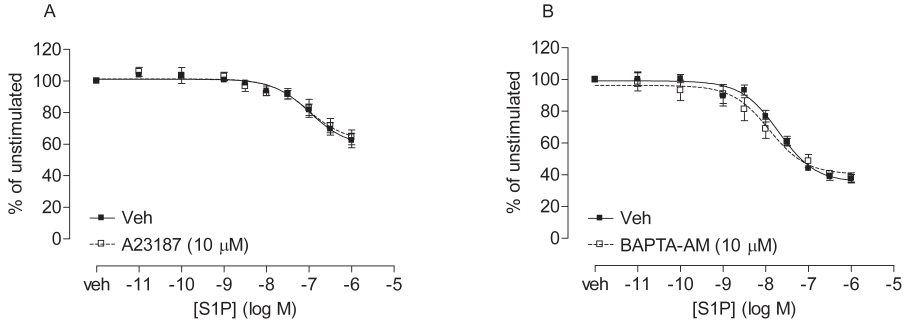


Figure 7. Influence of A23187 (10 µM) and BAPTA-AM (10 µM) on S1P-induced internalization of HisG-S1P$_1$ and HisG-S1P$_3$ receptor subtypes. Stimulations were carried out at 37 ºC for 30 min. Values are expressed as percentage of fluorescent signal measured for unstimulated cells and are presented as means ± s.e.m. of 3 independent experiments.

Discussion

Like most GPCRs, S1P receptors are succeptible to regulatory processes upon ligand stimulation. The interest for this mechanism has increased drastically since it has been the suggested mechanism of action for the immunosuppressive prodrug FTY720 (Gräler & Goetzl, 2004). We have used a previously reported, optimized method (Chapter 3) to compare $S1P_1$, $S1P_2$ and $S1P_3$ receptor internalization.

Internalization of the $S1P_1$ receptor has been previously studied (Liu et al., 1999) using a C-terminal GFP-tagged $S1P_1$ receptor in HEK293 cells. Interestingly, the data obtained in that study and the present study for the $S1P_1$ receptor are in good agreement. The time to internalize half of the $S1P_1$ receptors ($t_{½}$) is comparable (~15 min). In addition, resensitization was also comparable, reaching about 80% of the pretreatment level of receptors at 2 h after S1P washout. This confirms that S1P receptor internalization can be validly studied using our newly developed method.

Comparing internalization of the three S1P receptors ubiquitously expressed in the cardiovascular system, namely the $S1P_1$, $S1P_2$ and $S1P_3$ receptor (Alewijnse et al., 2004), revealed some interesting differences between the three receptor subtypes. While time-dependent S1P-induced internalization for the HisG-$S1P_1$ and HisG-$S1P_2$ receptor was comparable, the HisG-$S1P_3$ receptor was internalized much faster. In addition, the potency of S1P to induce internalization was significantly higher at the HisG-$S1P_3$ receptor compared to the other two subtypes. Furthermore, S1P-stimulation reduced membrane expression of HisG-$S1P_3$ receptor to lower levels than the HisG-$S1P_2$ receptor, which was in turn reduced to lower levels than the HisG-$S1P_1$ receptor. Finally, resensitization of the HisG-$S1P_3$ receptor to the membrane was slower than for the HisG-$S1P_1$ and HisG-$S1P_2$ receptor. Different S1P receptor subtypes activate different signaling pathways (Spiegel & Milstien, 2000). Could this be underlying the observed differences in internalization? When comparing signaling, the HisG-$S1P_1$ receptor inhibits cAMP accumulation, while the small S1P-induced increases in $[Ca^{2+}]_i$ seen in cells expressing the HisG-$S1P_1$ receptor are most mediated via βγ-subunits of G_i, although an effect of endogenous S1P receptors is also possible (Holdsworth et al., 2005). The HisG-$S1P_2$ receptor induces elevations in $[Ca^{2+}]_i$ and surprisingly elevations of cAMP accumulation, even though the $S1P_2$ receptor is generally reported to activate G_i-proteins. This effect has been observed before in CHO cells overexpressing the $S1P_2$ receptor, but has not yet been fully understood (Windh et al., 1999). Stimulation of the HisG-$S1P_3$ receptor results in both inhibition of cAMP accumula-

tion and elevations of $[Ca^{2+}]_i$. Interestingly, pre-treatment with PTX, an inhibitor of G_i-activation, shifted the S1P-induced internalization curve of the HisG-$S1P_3$ receptor to the right, whereas it did not affect S1P-induced internalization of the HisG-$S1P_1$ and HisG-$S1P_2$ receptor. Thus, activation of the G-protein influences internalization of the $S1P_3$ receptor, but not the $S1P_1$ or $S1P_2$ receptor.

We investigated internalization of these receptors in more detail by modulating both G_i and G_q-coupled signaling pathways. Incubations with Rp-cAMPs, an inhibitor of PKA, did not alter internalization. Thus, although inhibition of G_i-activation by PTX treatment showed an effect on internalization, inhibition of the downstream target PKA is not involved. Since there is no commercially available inhibitor of G_q-activation, we inhibited downstream signaling using inhibitors of the enzymes activated by the G_q-signaling cascade (U73122 for PLC and calphostin-C for PKC). Incubation with either inhibitor did not have any effect on S1P-induced internalization of any of the three S1P receptor subtypes. Thus, inihibiting the G_q-pathway at different levels has no influence on S1P-induced receptor internalization.

To study whether the $[Ca^{2+}]_i$, which is increased via $S1P_3$ receptor activation but not via $S1P_1$ receptor activation, influenced internalization, we applied the calcium ionophore A23187 to HisG-$S1P_1$ expressing cells to increase $[Ca^{2+}]_i$ and applied a calcium chelator BAPTA-AM to HisG-$S1P_3$ expressing cells to eliminate a possible effect on $[Ca^{2+}]_i$. Both approaches did not show any influence on S1P-induced receptor internalization of either subtypes. This means that changes in $[Ca^{2+}]_i$ are not involved in the differences seen between S1P-induced internalization of the S1P receptor subtypes.

We thus conclude that for the HisG-$S1P_3$ receptor, G_i-activation is of influence on S1P-induced internalization, but PKA, which is downstream in the G_i-pathway, does not have any effect. Influence of G_i-sensitive pathways in receptor internalization is also seen in for example α_{2A} and α_{2B}-adrenergic receptors, although partially (Olli-Lahdesmaki et al., 2004) or neuropeptide Y receptors (Pheng et al., 2003). Furthermore, although there are no available inhibitors of G_q-activation, none of the enzymes in this pathway, i.e. PLC and PKC seem to have any influence in HisG-$S1P_3$ receptor internalization. However, even though downstream signaling is not of influence, a direct effect of G_q-activation itself on S1P receptor internalization can not yet be ruled out. In addition, simulating the activation of both the G_q and G_i-pathways by increasing or decreasing $[Ca^{2+}]_i$ does not affect internalization of HisG-$S1P_1$ or HisG-$S1P_3$.

The data described in this article show a clear difference in the internalization of the HisG-$S1P_1$, HisG-$S1P_2$ and HisG-$S1P_3$ receptor subtypes. In part, these differences can be linked to a difference in signaling pathways activated by specific receptor stimulation, since G_i, but not G_q-activation seems to influence HisG-$S1P_3$ receptor internalization. However, this does not fully account for the observed differences. An additional explanation may be found in a recent investigation which showed that in contrast to $S1P_1$, the $S1P_3$ receptor internalization is independent of receptor phosphorylation (Rutherford et al., 2005). The observed differences in S1P receptor regulation could have implications for the functioning of drugs targeted at S1P receptors including FTY720.

References

Alewijnse, AE, Peters, SL, Michel, MC (2004). Cardiovascular effects of sphingosine-1-phosphate and other sphingomyelin metabolites. *Br J Pharmacol* **143**:666-684

Anliker, B, Chun, J (2004). Cell surface receptors in lysophospholipid signaling. *Semin Cell Dev Biol* **15**: 457-465

Bünemann, M, Hosey, MM (1999). G-protein coupled receptor kinases as modulators of G-protein signaling. *J Physiol* **517**: 5-23

Chun, J, Goetzl, EJ, Hla, T, Igarashi, Y, Lynch, KR, Moolenaar, W, et al. (2002). International Union of Pharmacology. XXXIV. Lysophospholipid receptor nomenclature. *Pharmacol Rev* **54**: 265-269

Gräler, MH, Goetzl, EJ (2004). The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G-protein-coupled receptors. *FASEB J* **18**:551-553

Holdsworth, G, Slocombe, P, Hutchinson, G, Milligan, G (2005). Analysis of endogenous S1P and LPA receptor expression in CHO-K1 cells. *Gene* **350**: 59-63

Kappos, L, Antel, J, Comi, G, Montalban, X, O'Connor, P, Polman, CH, et al. (2006). Oral fingolimod (FTY720) for relapsing multiple sclerosis. *N Engl J Med* **355**:1124-1140

Liu, CH, Thangada, S, Lee, MJ, Van Brocklyn, JR, Spiegel, S, Hla, T (1999). Ligand-induced trafficking of the sphingosine-1-phosphate receptor EDG-1. *Mol Biol Cell* **10**:1179-1190

Mandala, S, Hajdu, R, Bergstrom, J, Quackenbush, E, Xie, J, Milligan, J, et al. (2002). Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. *Science* **296**:346-349

Matloubian, M, Lo, CG, Cinamon, G, Lesneski, MJ, Xu, Y, Brinkmann, V, et al. (2004). Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature* **427**: 355-360

Okajima, F (2002). Plasma lipoproteins behave as carriers of extracellular sphingosine 1-phosphate: is this an atherogenic mediator or an anti-atherogenic mediator? *Biochim Biophys Acta* **1582**:132-137

Olli-Lahdesmaki, T, Tiger, M, Vainio, M, Scheinin, M, Kallio, J (2004). Ligand-induced alpha2-adrenoceptor endocytosis: relationship to Gi protein activation. *Biochem Biophys Res Commun* **321**: 226-233

Pappu, R, Schwab, SR, Cornelissen, I, Pereira, JP, Regard, JB, Xu, Y, et al. (2007). Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. *Science* **316**:295-298

Pheng, LH, Dumont, Y, Fournier, A, Chabot, JG, Beaudet, A, Quirion, R (2003). Agonist- and antagonist-induced sequestration/internalization of neuropeptide Y Y1 receptors in HEK293 cells. *Br J Pharmacol* **139**: 695-704

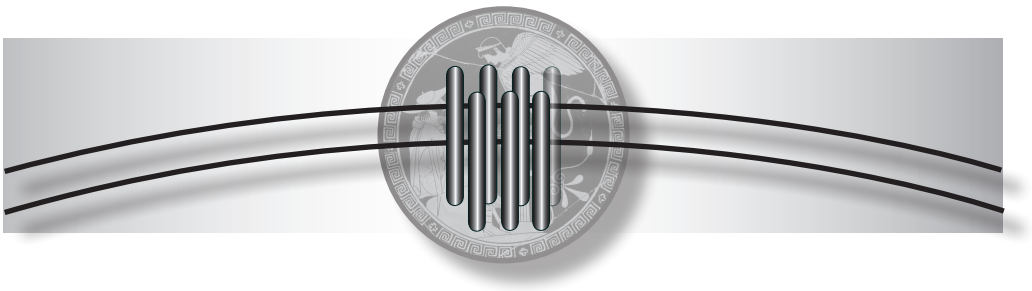
Rutherford, C, Ord-Shrimpton, FU, Sands, WA, Pediani, JD, Benovic, JL, McGrath, JC, et al. (2005). Phosphorylation-independent internalization and desensitization of the human sphingosine-1-phosphate receptor S1P3. *Cell Signal* **17**: 997-1009

Spiegel, S, Milstien, S (2000). Functions of a new family of sphingosine-1-phosphate receptors. *Biochim Biophys Acta* **1484**:107-116

Spiegel, S, Milstien, S (2003). Sphingosine-1-phosphate: an enigmatic signaling lipid. *Nat Rev Mol Cell Biol* B:397-407

von Zastrow M (2001). Endocytosis and downregulation of G protein-coupled receptors. *Parkinsonism Relat Disord* **7**:265-271

Windh, RT, Lee, MJ, Hla, T, An, S, Barr, AJ, Manning, DR (1999). Differential coupling of the sphingosine 1-phosphate receptors Edg-1, Edg-3, and H218/Edg-5 to the G(i), G(q), and G(12) families of heterotrimeric G proteins. *J Biol Chem* **274**:27351-27358



Involvement of the third amino acid of the D/ERY motif in G-protein selectivity of S1P receptors

Chapter 6

Involvement of the third amino acid of the D/ERY motif of S1P receptors in G-protein selectivity

Manuscript in preparation

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Abstract

The highly conserved D/ERY motif is known to play an important role in G-protein coupling and/or constitutive activity of G-protein coupled receptors (GPCRs). However, the specific amino acid residues that are important in G-protein selectivity and which for instance restrict some GPCRs from coupling to multiple G-proteins are not exactly known. Among sphingosine-1-phosphate (S1P) receptors only the $S1P_1$ receptor, which couples to $G_{i/o}$-proteins only, contains an ERY motif whereas the other S1P receptor subtypes show some variance in the third amino acid of this motif. In this paper we investigated the role of the third amino acid residue of the D/ERY motif in regulating the preference for certain signaling responses, we compared wild-type and mutant S1P receptors. Mutating the ERY motif of the $S1P_1$ receptor to ERH, the motif present in the wild-type $S1P_2$ and $S1P_3$ receptor, or ERA reduced S1P-induced G_i-mediated signaling without inducing calcium elevations. The G_i-mediated responses of the $S1P_1$ specific agonist SEW2871 were also decreased at both mutant receptors, whereas the effects of FTY720-P at both mutant receptors were unchanged suggesting that these compounds induce different conformational states of the receptor. Mutating the ERH motif of the $S1P_2$ or $S1P_3$ receptor to an ERY or ERA motif did not induce major changes in signaling of these receptors, except for an S1P-induced increase in cAMP accumulation in the $S1P_2$ mutant receptor containing an ERY motif.

We conclude that the tyrosine residue of the D/ERY motif does not restrict the $S1P_1$ receptor from activating multiple G-proteins; however, mutating this residue revealed differences in response patterns induced by either S1P, SEW2871 or FTY720-P.

Introduction

The bioactive sphingolipid sphingosine-1-phosphate (S1P) regulates many different cellular functions including proliferation, migration and differentiation via a family of specific G-protein coupled receptors (GPCRs). This family currently consists of five subtypes ($S1P_{1-5}$), of which the $S1P_1$, $S1P_2$ and $S1P_3$ receptors are ubiquitously expressed, whereas the $S1P_4$ receptor is mainly found in lymphoid tissue and the $S1P_5$ receptor in the brain (Anliker & Chun, 2004).

GPCRs exert their signals primarily via activation of heterotrimeric G-proteins, of which there are four subfamilies, namely $G_{i/o}$, G_s, $G_{q/11}$ and $G_{12/13}$ (for review see Hermans, 2003). With 28 α subunits, six β and 12 γ-subunits being described (Hermans, 2003; Carbrea-Vera et al., 2003), many different combinations are possible, resulting in a diversity of pathways to activate.

Most GPCRs couple preferentially to one subfamily of G-proteins (89 % of the extensive group reviewed by Wong, 2003). However, a small fraction of GPCRs shows promiscuous coupling as they can activate multiple subfamilies of G-proteins. At present, it is not precisely known what restricts GPCRs from coupling to multiple G-proteins. It has been suggested that a specific amino acid region situated at the boundary of the third transmembrane domain and the second intracellular loop of the GPCR may play a role in G-protein selectivity. This region contains a triplicate of amino acids; D/ERY (Asp/Glu-Arg-Tyr), which is highly conserved among class A GPCRs (for 86 %, 97 % and 67 % respectively) (Flanagan, 2005). Indeed, several studies have found this D/ERY motif to be involved in constitutive receptor activity or G-protein coupling based upon site-directed mutagenesis within this motif (Capra et al., 2004; Favre et al., 2005; et al., 2006). Most of these studies have been done on the aspartate/glutamic acid and/or arginine residue while the tyrosine residue has been much less investigated, possibly due to the fact that is the least conserved of the three. The effect of mutations in the D/ERY motif seems to differ between receptors or receptor families (reviewed in Rovati et al., 2007). It has been suggested that within the class A GPCRs, to which the S1P receptors belong, there may be two groups that differently use the D/ERY motif (Capra et al., 2004). In one group this motif is involved in keeping the receptor in an inactive ground state. In this group, which includes the α_{1B}-adrenoceptor and the histamine H_2 receptor, mutations in this motif do not affect ligand binding, but result in an increase or induction of constitutive receptor activity, sometimes accompanied by an increase in agonist affinity. In the second group of receptors, which includes

the α_{2A}-adrenoceptor and M_1 muscarinic acetylcholine receptor, mutations in the D/ERY motif result in changes in agonist-induced responses without inducing constitutive activity.

Unlike most GPCR, four out of five members of the S1P receptor family can couple to and activate multiple G-protein subfamilies. The $S1P_1$ receptor is the only receptor which exclusively activates G_i-protein coupled pathways (Ancellin & Hla, 1999). The $S1P_2$ and $S1P_3$ receptor activate G_i, G_q and $G_{12/13}$-proteins (Ancellin & Hla, 1999) and the $S1P_4$ and $S1P_5$ receptor activate G_i and $G_{12/13}$-proteins (Im et al., 2000; Malek et al., 2001; Gräler et al., 2003). Interestingly, the $S1P_1$ receptor is also the only subtype that contains a tyrosine residue, as found in most GPCRs at the third position of the D/ERY motif. The $S1P_2$, $S1P_3$, $S1P_4$ and $S1P_5$ receptors contain either a histidine ($S1P_2$ and $S1P_3$), or phenylalanine ($S1P_4$) or a serine ($S1P_5$) at this position. From these observations, it may thus be hypothesized that the tyrosine residue may contribute to restricting the $S1P_1$ receptor from promiscuous coupling.

In this study we, therefore, investigated whether this third amino acid is indeed important in G-protein selectivity by mutating the ERY motif of the $S1P_1$ receptor to an ERH (as present in the $S1P_2$ and $S1P_3$ receptor) or an ERA motif (generating a "neutral" mutation). Chinese hamster ovary (CHO)-FlpIn cells were transfected with either the wild-type (wt) or mutant receptors, and S1P-induced changes in $[Ca^{2+}]_i$ and cAMP accumulation (typical G_q and G_i-coupled pathways) in these cell lines were subsequently studied. Besides the natural ligand S1P, a selective $S1P_1$ agonist (SEW2871) and the phosphorylated form of the new immunosuppressive prodrug FTY720, an agonist for the $S1P_{1,3-5}$ receptor were used (Mandala et al., 2002).

Material & Methods

Materials

pcDNA3.1 containing the entire coding region of the human $S1P_1$, $S1P_2$ or $S1P_3$ receptor was purchased from UMR cDNA Resource Center (Rolla, MO, USA). Cell culture media, hygromycine B, Lipofectamine™ 2000, Optimem, Hank's Balanced Salt Solution (HBSS), pOG44, pcDNA3.1/HisA/C, pcDNA5/FRT/TO and CHO-FlpIn cells, were obtained from Invitrogen (Breda, The Netherlands). Pluronic acid and Fluo-4 AM were obtained from Molecular Probes (via Invitrogen). Restriction enzymes (ApaI, BamHI, XhoI, EcoRI and HindIII) were obtained

from Fermentas Life Sciences (St. Leon-Rot, Germany). Foetal calf serum (FCS), enzyme free cell dissociation buffer and penicillin/streptomycin were obtained from Gibco (via Invitrogen). LANCE™ cAMP 384 kit and white 384 well optiplates were obtained from Perkin Elmer (Zaventem, Belgium). S1P was obtained from Avanti-Polar Lipids, Inc (via Instuchemie B.V., Delfzijl, The Netherlands). Probenecid, HEPES, EGTA, Triton X-100, 3-isobutyl-1-methylxanthine, bovine serum albumin (BSA) (fatty acid free) and activated charcoal were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). Black, clear bottom 96 well plates were obtained from Greiner Bio One (Alphen aan den Rijn, The Netherlands). 5-[4-Phenyl-5-(trifluoromethyl)-2-thienyl]-3-[3-(trifluoromethyl)phenyl]-1,2,4-oxadiazole (SEW2871) was obtained from Cayman Chemical Company (Ann Arbor, MI, USA). 2-Amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol mono(dihydrogen phosphate) ester (FTY720-P) was synthesized according to previously described methods (Albert et al., 2005).

Molecular cloning and transfection

A N-terminal HisG-tag was added to the $S1P_{1-3}$ receptors via cloning into pcDNA3.1/HisC using BamHI&XhoI (for $S1P_1$) or BamHI&ApaI (for $S1P_2$) or into pcDNA3.1/HisA using BamHI&XhoI (for $S1P_3$). A second cloning step was done using HindIII&XhoI (for $S1P_{1,3}$) or HindIII&ApaI (for $S1P_2$) to clone the HisG-tagged S1P receptors into the expression vector pcDNA5/FRT/TO.

Mutations in the ERY/H motif of the $S1P_{1-3}$ receptor were made via site directed mutagenesis using the primers listed in table 1. All constructs were confirmed by sequencing of the plasmids and the stable cell lines.

Transfection of plasmids into CHO-FlpIn cells and clonal selection was done as described previously (Chapter 2). Membrane expression of the receptors was confirmed using an immunocytochemical method to detect membrane receptors by detecting the N-terminal HisG-tag as described previously (Chapter 3). The cDNA of the $S1P_3$ receptor obtained from Guthrie contained a point mutation ($G^{962}A$ which results in $R^{321}Q$) which was removed by PCR using the following primers (forward: TGCCTGGTCAGGGGGCGGGGGGCCCG, reverse: CGGGCCCCCCGCCCCCTGACCAGGCA). The $S1P_3$ receptor coding DNA in the resulting plasmid as well as the stable cell line constructed with this plasmid have been confirmed by sequencing.

Table 1. Primers used for mutations in the ERY/H motif of the $S1P_{1-3}$ receptors via site directed mutagenesis.

Receptor + Mutation	Primer
$S1P_1$ $Y^{143}A$	Forward: CATCGCCATTGAGCGCGCAATCACAATGCT
	Reverse: GCGCTCAATGGCGATGGCGAGGAGACTG
$S1P_1$ $Y^{143}H$	Forward : CGCCATTGAGCGCCATATCACAATG
	Reverse : CATTGTGATATGGCGCTCAATGGCG
$S1P_2$ $H^{131}A$	Forward : ATCGCCATTGAGCGCGCAGTGGCCATTGCC
	Reverse : GCGCTCAATGGCGATGGCCAGGAGGCTGAA
$S1P_2$ $H^{131}Y$	Forward : CCATTGAGCGCTACGTGGCCATTGC
	Reverse : GCAATGGCCACGTAGCGCTCAATGG
$S1P_3$ $H^{137}A$	Forward : GCCATCGAGCGGGCCTTGACAATGAT
	Reverse : ATCATTGTCAAGGCCCGCTCGATGGC
$S1P_3$ $H^{137}Y$	Forward : GCCATCGAGCGGTACTTGACAATGA
	Reverse : TCATTGTCAAGTACCGCTCGATGGC

Cell culture

CHO-FlpIn cells stably expressing the $S1P_1$, $S1P_2$ or $S1P_3$ wt or mutant receptors were passaged 1:5 every two or three days in F-12 Nutrient Mixture (Ham) with L-glutamine, supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 313 µg/ml hygromycin B and 10 % charcoal-stripped FCS. All cell lines were cultured at 37 °C in humidified air containing 5 % CO_2.

cAMP assay

The LANCE™ cAMP 384 kit was used to determine cAMP concentrations according to the manufacturer's protocol. CHO-FlpIn cells, after overnight serum-starvation, were detached from the surface using dissociation buffer. Cells were washed once with HBSS and subsequently resuspended in stimulation buffer, containing HBSS with 0.05% BSA (fatty-acid free) and 5 mM HEPES. Stimulation mixtures consisted of stimulation buffer with 1 mM (final concentration 0.5 mM) 3-isobutyl-1-methylxanthine, 6 µM (final concentration 3 µM) forskolin and the concentration response curve of the indicated compounds. Cells were added to the stimulation mixtures 1:1 in a 384 well optiplate at 2500 cells/well and stimulated for 60 minutes at room temperature with a total volume of 20 µl/well. Detection of the cAMP formed during stimulation was done according to the manufacturer's protocol. Measurements were done on a microplate reader (Victor 2, Wallac, Perkin Elmer) 16-24 hours after adding detection buffer and antibody mixture.

Intracellular calcium measurement

Intracellular calcium measurements were performed as previously described (Chapter 2) with minor changes. CHO-FlpIn cells were plated in a black, clear bottom 96 wells plate at 40,000 cells/well. After one day growth, cells were serum starved overnight. Cells were then loaded for 1 hour with basic buffer (HBSS containing 20 mM HEPES, 2.5 mM probenecid) containing 4 µM Fluo-4 AM ester and 0.42 % v/v pluronic acid and incubated at 37 °C. After loading, cells were washed twice with basic buffer and incubated at 37 °C with basic buffer for 60 min The fluorescence signal was measured at basal level, followed by ligand stimulation, Triton (5 % v/v) addition (resulting in F_{max}) and 250 mM EGTA addition (resulting in F_{min}). The $[Ca^{2+}]_i$ was calculated via the equation: $[Ca^{2+}]_i = K_d * ((F-F_{min})/(F_{max}-F))$. K_d being the dissociation constant of the binding of Fluo-4 to calcium (345 nM). The increase in $[Ca^{2+}]_i$ upon ligand stimulation was calculated as the difference between the $[Ca^{2+}]_i$ for the basal level and after adding a ligand.

Data analysis

Concentration response curves were analyzed by fitting sigmoidal functions to the experimental data using Prism 4 (Graphpad Software, Inc., San Diego, CA, USA). Data are expressed as means ± s.e.m. One-way ANOVA with a Dunnett's correction or Student's t-test was applied where appropriate. $P<0.05$ was considered significant.

Results

Effect of mutations in the ERY motif of the $S1P_1$ receptor on signaling

In cells expressing the wt $S1P_1$ receptor S1P inhibited forskolin-induced cAMP accumulation. At both, the $S1P_1$ $Y^{143}A$ and $S1P_1$ $Y^{143}H$ mutant receptors, the potency of S1P to inhibit forskolin-induced cAMP accumulation was significantly reduced (wt pEC_{50} = 9.7 ± 0.1 (n=8); $Y^{143}A$ pEC_{50} = 7.8 ± 0.2 (n=4); $Y^{143}H$ pEC_{50} = 8.9 ± 0.3 (n=3); $p<0.05$)) (Figure 1A). This difference was not seen upon stimulation with FTY720-P (wt pEC_{50} = 10.2 ± 0.1 (n=9); $Y^{143}A$ pEC_{50} = 10.5 ± 0.2 (n=5); $Y^{143}H$ pEC_{50} = 9.8 ± 0.1 (n=4)) (Figure 1B). The potency of the $S1P_1$ specific agonist SEW2871 to inhibit forskolin-induced cAMP accumulation was significantly decreased at the $S1P_1$ $Y^{143}A$ and the $S1P_1$ $Y^{143}H$ mutant receptor compared to the wt $S1P_1$ receptor (wt pEC_{50} = 8.2 ± 0.1 (n=12); $Y^{143}A$ pEC_{50} = 7.5 ± 0.3 (n=7); $Y^{143}H$ pEC_{50} = 7.7 ± 0.1 (n=4) $p<0.05$) (Figure 1C).

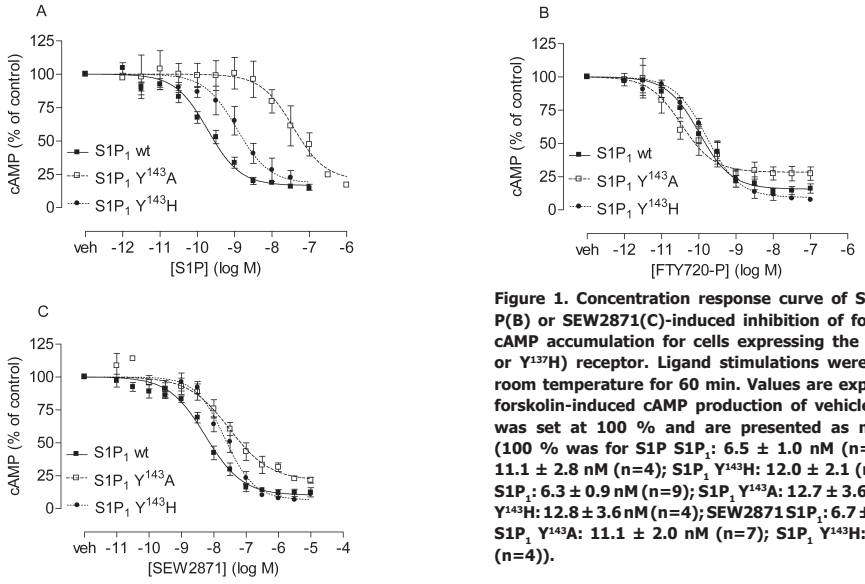


Figure 1. Concentration response curve of S1P(A), FTY720-P(B) or SEW2871(C)-induced inhibition of forskolin-induced cAMP accumulation for cells expressing the $S1P_1$ (wt, $Y^{137}A$ or $Y^{137}H$) receptor. Ligand stimulations were carried out at room temperature for 60 min. Values are expressed as % of forskolin-induced cAMP production of vehicle control which was set at 100 % and are presented as means ± s.e.m. (100 % was for S1P $S1P_1$: 6.5 ± 1.0 nM (n=8); $S1P_1$ $Y^{143}A$: 11.1 ± 2.8 nM (n=4); $S1P_1$ $Y^{143}H$: 12.0 ± 2.1 (n=3); FTY720-P $S1P_1$: 6.3 ± 0.9 nM (n=9); $S1P_1$ $Y^{143}A$: 12.7 ± 3.6 nM (n=5); $S1P_1$ $Y^{143}H$: 12.8 ± 3.6 nM (n=4); SEW2871 $S1P_1$: 6.7 ± 0.9 nM (n=12); $S1P_1$ $Y^{143}A$: 11.1 ± 2.0 nM (n=7); $S1P_1$ $Y^{143}H$: 12.1 ± 1.7 nM (n=4)).

S1P, but not FTY720-P induced increases in $[Ca^{2+}]_i$ in mock transfected cells (pEC_{50} = 7.0 ± 0.3; E_{max} = 297 ± 57 nM (n=4)) (Figure 2). S1P and FTY720-P induced significantly higher increases in $[Ca^{2+}]_i$ in cells expressing the $S1P_1$ wt receptor (S1P: pEC_{50} = 7.1 ± 0.1; E_{max} = 857 ± 122 nM (n=6) $p<0.05$; FTY720-P: pEC_{50} = 6.2 ± 0.2; E_{max} = 614 ± 48 nM (n=9)). These characteristics for S1P and FTY720-P were not significantly changed for both $S1P_1$ mutant receptors ($S1P_1Y^{143}A$ and $S1P_1Y^{143}H$) from the $S1P_1$ wt receptor (**$S1P_1Y^{143}A$** S1P: pEC_{50} = 6.9 ± 0.1; E_{max} = 456 ± 32 nM (n=3); FTY720-P: pEC_{50} = 6.9 ± 0.1; E_{max} = 344 ± 56 nM (n=3); **$S1P_1Y^{143}H$** S1P: pEC_{50} = 7.4 ± 0.1; E_{max} = 824 ± 67 nM (n=7); FTY720-P: pEC_{50} = 6.6 ± 0.1; E_{max} = 705 ± 92 nM (n=9) (Figure 2)

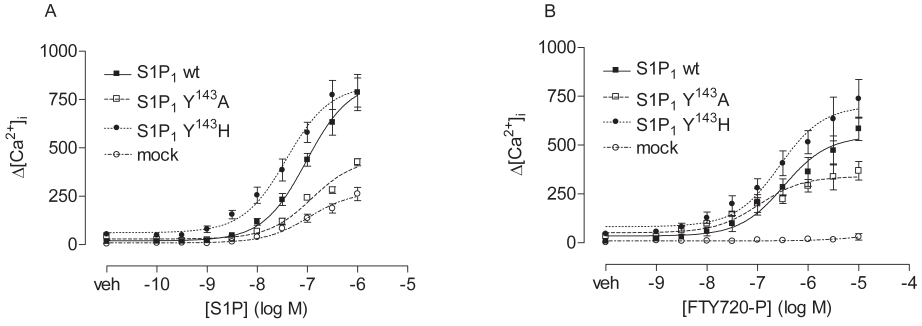


Figure 2. Concentration response curve of S1P(A) or FTY720-P(B)-induced elevations in $[Ca^{2+}]_i$ for cells expressing the $S1P_1$ (wt, $Y^{143}A$ or $Y^{143}H$) receptor or mock transfected cells. Values are mean ± s.e.m. (n=3-9).

Effect of mutation in the ERH motif of the $S1P_2$ receptor on signaling

In CHO-FlpIn cells expressing the wild type $S1P_2$ receptor, S1P slightly increased forskolin-induced cAMP accumulation (E_{max} =139 ± 19 % of basal (n=6)) (Figure 3A). A comparable S1P response was observed in cells expressing the $S1P_2$ $H^{131}A$ mutant receptor (E_{max} = 110 ±10 % of basal (n=6)). Interestingly, the S1P-induced increase in forskolin-induced cAMP accumulation was significantly greater for the $S1P_2$ $H^{131}Y$ mutant receptor compared to the wt receptor (E_{max} = 258 ± 28 % of basal (n=6) ($p<0.05$)) (Figure 3A).

In addition to the effects on cAMP accumulation, stimulation of the $S1P_2$ wt cells with S1P also resulted in a concentration dependent increase in $[Ca^{2+}]_i$ (pEC_{50} = 7.3 ± 0.1 (n=3); E_{max} = 1255 ± 400 nM (n=3)) (Figure 3B). This effect was not significantly altered in cells expressing the $S1P_2$ $H^{131}A$ or $S1P_2$ $H^{131}Y$ mutant receptor (Figure 3B). Because the S1P-induced elevations in $[Ca^{2+}]_i$ sometimes approach the effect observed for TritonX, which is used to determine the total concentration of calcium, in some cases (especially at high S1P concentrations) no reliable calculation of the change in $[Ca^{2+}]_i$ is possible.

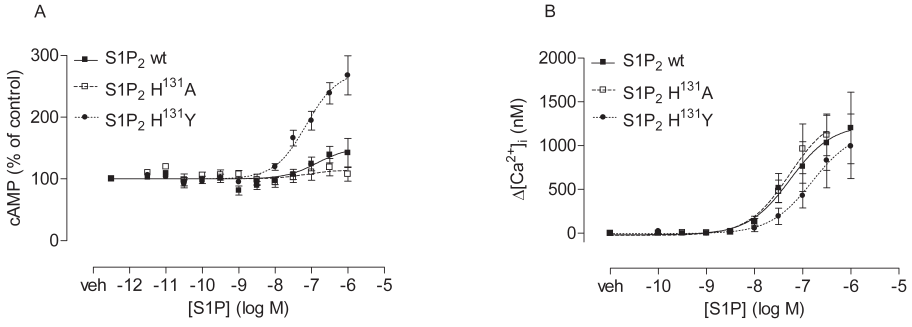


Figure 3. Concentration response curve of S1P-induced change in forskolin-induced cAMP accumulation for cells expressing the $S1P_2$ (wt, $H^{131}A$ or $H^{131}Y$) receptor. Ligand stimulations were carried out at room temperature for 60 min. Values are expressed as % of forskolin-induced cAMP production of vehicle control which was set at 100% and are presented as means ± s.e.m. (100% was for $S1P_2$: 4.8 ± 0.6 nM (n=6); $S1P_2$ $H^{131}A$: 5.4 ± 0.7 nM (n=6); $S1P_2$ $H^{131}Y$: 6.1 ± 0.5 (n=6)) (A). Concentration response curve of S1P-induced elevations in $[Ca^{2+}]_i$ for cells expressing the $S1P_2$ (wt, $H^{131}A$ or $H^{131}Y$) receptor. Values are mean ± s.e.m. (n=3) (B).

Effect of mutation in the ERH motif of the $S1P_3$ receptor on signaling

In cells expressing the wt $S1P_3$ receptor S1P and FTY720-P inhibited forskolin-induced cAMP accumulation in a concentration dependent manner (Figure 4). The concentration response curve for S1P or FTY720-P was not significantly altered for the $S1P_3$ $H^{137}A$ receptor or $S1P_3$ $H^{137}Y$ mutant receptor compared to the $S1P_3$ wt receptor (Figure 4, Table 2).

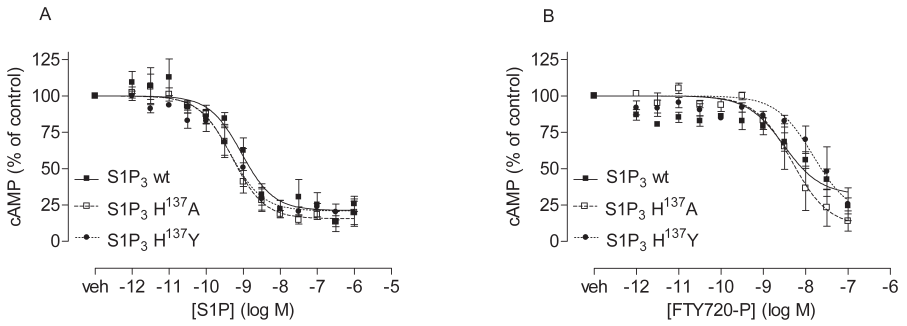


Figure 4. Concentration response curve of S1P(A) or FTY720-P(B)-induced inhibition of forskolin-induced cAMP accumulation for cells expressing the $S1P_3$ (wt, $H^{137}A$ or $H^{137}Y$) receptor. Ligand stimulations were carried out at room temperature for 60 min. Values are expressed as % of forskolin-induced cAMP production of vehicle control which was set at 100% and are presented as means ± s.e.m. (100% was for S1P $S1P_3$: 13.5 ± 2.7 nM (n=5); $S1P_3$ $H^{137}A$: 17.6 ± 4.5 nM (n=5); $S1P_3$ $H^{137}Y$: 12.6 ± 3.4 (n=5); FTY720-P $S1P_3$: 15.3 ± 2.0 nM (n=3); $S1P_3$ $H^{137}A$: 23.0 ± 1.1 nM (n=3); $S1P_3$ $H^{137}Y$: 22.4 ± 4.5 (n=3).

Both, S1P and FTY720-P, potently induced elevations in $[Ca^{2+}]_i$ in cells expressing the wt $S1P_3$ receptor (Figure 5). This potency was only significantly increased for the $S1P_3$ $H^{137}A$ receptor compared to the wt upon stimulation with FTY720-P (Figure 5). Also in these experiments, the data could in some cases not be reliably calculated as described for the $S1P_2$ receptors.

Table 2. pEC_{50} and maximal inhibition values for the $S1P_3$ wt and mutant receptors in ligand-induced inhibition of forskolin-induced cAMP accumulation. * p<0.05 compared to wt.

ligand		wt	$H^{137}A$	$H^{137}Y$
S1P	pEC_{50}	9.0 ± 0.2 (5)	9.5 ± 0.1 (5)	9.1 ± 0.3 (5)
	E_{max}	71 ± 8 %	80 ± 4 %	78 ± 3 %
FTY720-P	pEC_{50}	8.2 ± 0.1 (3)	8.7 ± 0.1 (3)*	8.0 ± 0.1 (3)
	E_{max}	88 ± 4 %	94 ± 4 %	81 ± 2 %

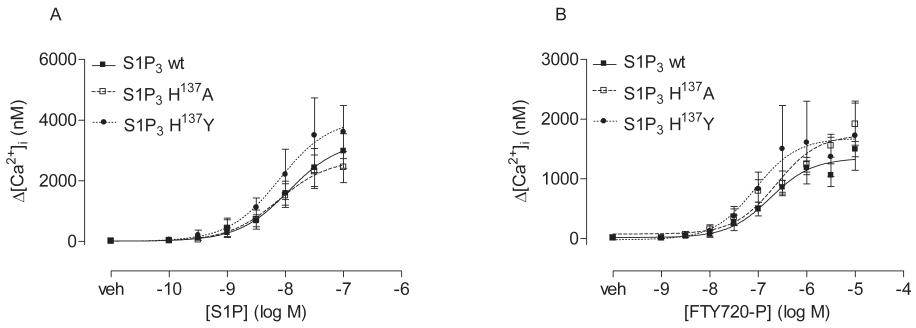


Figure 5. Concentration response curve of S1P(A) or FTY720-P(B)-induced elevations in $[Ca^{2+}]_i$ for cells expressing the $S1P_3$ (wt, $H^{137}A$ or $H^{137}Y$) receptor. Values are mean ± s.e.m. (n=3-4).

Discussion

The specific molecular determinants of GPCRs controlling their coupling specificity to G-proteins have remained elusive. However, for some receptors, the D/ERY motif has been shown to be involved in G-protein coupling and activation (Favre et al., 2005; Capra et al., 2004; Rovati et al., 2007). For the family of S1P receptors, the third amino acid residue of this motif shows a remarkable variance, possibly correlating with the selectivity of the S1P receptor subtypes for specific G-proteins or the lack thereof. The $S1P_1$ receptor contains an ERY motif and couples exclusively to G_i-proteins. The other four subtypes show promiscuous coupling to G_i, G_q and $G_{12/13}$-proteins ($S1P_2$ and $S1P_3$) or G_i and $G_{12/13}$ proteins ($S1P_4$ and $S1P_5$) and contain either an ERH ($S1P_2$ and $S1P_3$), ERF ($S1P_4$) or ERS ($S1P_5$) motif.

Mutating the ERY motif of the $S1P_1$ receptor to an ERH motif, which is present in the $S1P_2$ and $S1P_3$ receptor, showed an interesting change in signal transduction. Both, at the $S1P_1$ $Y^{143}H$ and $Y^{143}A$ mutant receptor the potency of S1P and SEW2871 to inhibit forskolin-induced cAMP accumulation was significantly decreased, which points in the direction of a reduced coupling to and activation of G_i-proteins. Similar effects were observed when mutating the tyrosine residue of the cannabinoid receptor CB_2, which is phylogenetically related to S1P receptors (Rhee et al., 2000; Alberich et al., 2004). In both studies, the DRY motif was mutated to a DRA motif and a loss of signaling to the G_i coupled pathway was observed.

Remarkably, the decrease in G_i-signaling of the $S1P_1$ $Y^{143}H$ and $Y^{143}A$ mutant receptor was not observed when using FTY720-P instead of S1P or SEW2871. Mutating the tyrosine residue of the ERY motif of the $S1P_1$ receptor thus seems to differentially affect the signaling of the ligands. Differential effects between S1P and SEW2871 on the one hand and FTY720-P on the other have also been observed when studying receptor fate after internalization. In that study $S1P_1$ receptors internalized upon stimulation with S1P or SEW2871 were shown to recycle back to the plasma membrane, while $S1P_1$ receptor internalized upon FTY720-P stimulation were broken down in lyzosomes (Oo et al., 2007). The differences in receptor fate after internalization were assigned to polyubiquitinylation of the $S1P_1$ receptor by FTY720, but not by S1P (Oo et al., 2007). These results together with the results obtained in this study strongly suggest differences in the ligand-induced $S1P_1$ receptor conformation between S1P and SEW2871 versus FTY720-P.

The increases in $[Ca^{2+}]_i$ in CHO-FlpIn $S1P_1$ cells induced by both, S1P and FTY720-P were significantly different from the responses observed in mock-

transfected cells. Because the S1P and FTY720-P induced changes in $[Ca^{2+}]_i$ seem to be decreased for the $S1P_1Y^{143}A$ mutant receptor, which also showed decreased G_i-signaling, the calcium effects are most likely G_i-mediated. However, definitive proof to confirm that the observed calcium effects are indeed G_i-mediated is still missing.

In contrast to the effects of mutations in the ERY motif observed for the $S1P_1$ receptor, the $S1P_2$ and $S1P_3$ receptors did not show many differences in G_i or G_q-activation when the ERH motif was mutated into ERY or ERA. No changes were seen for S1P and/or FTY720-P-induced inhibition of forskolin-induced cAMP accumulation or increases in $[Ca^{2+}]_i$ besides one remarkable observation. S1P increased forskolin-induced cAMP accumulation via the $S1P_2$ receptor rather than decreased, as one would expect based upon activation of G_i-proteins. Such an effect has been reported before in CHO cells (Windh et al., 1999), and was shown not to be induced via $[Ca^{2+}]_i$ (Kon et al., 1999). The authors suggest that it may be due to an inhibition of phosphodiesterase activity which results in less break-down of cAMP. This is, however, unlikely in our system, since the use of IBMX should inhibit all phosphodiesterase activity. The observed increase in cAMP was even more pronounced in the $S1P_2$ $H^{131}Y$ mutant receptor.

Overall, we conclude that mutations in the third amino acid of the D/ERY motif of the S1P receptor regulate G-protein mediated signaling, possibly via influencing the conformational state of the receptor. This could then clarify the observed differences in G_i-signaling in response to S1P and SEW2871 compared to FTY720-P. However, if the third amino acid is a tyrosine, it does not restrict the receptor from promiscuous coupling or influence constitutive receptor activity as seen for some other GPCRs.

References

Alberich Jordà M, Rayman N, Tas M, Verbakel SE, Battista N, van Lom K, Löwenberg B, Maccarrone M, Delwel R (2004). The peripheral cannabinoid receptor Cb2, frequently expressed on AML blasts, either induces a neutrophilic differentiation block or confers abnormal migration properties in a ligand-dependent manner. *Blood* **104**:526-534

Albert R, Hinterding K, Brinkmann V, Guerini D, Muller-Hartwieg C, Knecht H, Simeon C, Streiff M, Wagner T, Welzenbach K, Zecri F, Zollinger M, Cooke N, Francotte E (2005). Novel immunomodulator FTY720 is phosphorylated in rats and humans to form a single stereoisomer. Identification, chemical proof, and biological characterization of the biologically active species and its enantiomer. *J Med Chem* **48**:5373-5377

Ancellin N, Hla T (1999). Differential pharmacological properties and signal transduction of the sphingosine 1-phosphate receptors EDG-1, EDG-3, and EDG-5. *J Biol Chem* **274**:18997-9002

Anliker B, Chun J (2004). Cell surface receptors in lysophospholipid signaling. *Semin Cell Dev Biol* **15**:457-465

Capra V, Veltri A, Foglia C, Crimaldi L, Habib A, Parenti M, Rovati GE (2004). Mutational analysis of the highly conserved ERY motif of the thromboxane A2 receptor: alternative role in G protein-coupled receptor signaling. *Mol Pharmacol* **66**:880-889

Cabrera-Vera TM, Vanhauwe J, Thomas TO, Medkova M, Preininger A, Mazzoni MR, Hamm HE. (2003) Insights into G protein structure, function, and regulation. *Endocr Rev* **24**:765-781

Favre N, Fanelli F, Missotten M, Nichols A, Wilson J, di Tiani M, Rommel C, Scheer A (2005). The DRY motif as a molecular switch of the human oxytocin receptor. *Biochemistry* **44**:9990-10008

Flanagan CA (2005). A GPCR that is not "DRY". *Mol Pharmacol* **68**:1-3

Gräler MH, Grosse R, Kusch A, Kremmer E, Gudermann T, Lipp M (2003). The sphingosine 1-phosphate receptor S1P4 regulates cell shape and motility via coupling to G_i and $G_{12/13}$. *J Cell Biochem* **89**:507-519

Hermans E (2003). Biochemical and pharmacological control of the multiplicity of coupling at G-protein-coupled receptors. *Pharmacol Ther* **99**:25-44

Im DS, Heise CE, Ancellin N, O'Dowd BF, Shei GJ, Heavens RP, Rigby MR, Hla T, Mandala S, McAllister G, George SR, Lynch KR (2000). Characterization of a novel sphingosine 1-phosphate receptor, Edg-8. *J Biol Chem* **275**:14281-14286

Kon J, Sato K, Watanabe T, Tomura H, Kuwabara A, Kimura T, Tamama K, Ishizuka T, Murata N, Kanda T, Kobayashi I, Ohta H, Ui M, Okajima F (1999). Comparison of intrinsic activities of the putative sphingosine 1-phosphate receptor subtypes to regulate several signaling pathways in their cDNA-transfected Chinese hamster ovary cells. *J Biol Chem* **274**:23940-23947

Malek RL, Toman RE, Edsall LC, Wong S, Chiu J, Letterle CA, Van Brocklyn JR, Milstien S, Spiegel S, Lee NH (2001). Nrg-1 belongs to the endothelial differentiation gene family of G protein-coupled sphingosine-1-phosphate receptors. *J Biol Chem* **276**:5692-5699

Mandala S, Hajdu R, Bergstrom J, Quackenbush E, Xie J, Milligan J, Thornton R, Shei GJ, Card D, Keohane C, Rosenbach M, Hale J, Lynch CL, Rupprecht K, Parsons W, Rosen H (2002). Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. *Science* **296**:346-349

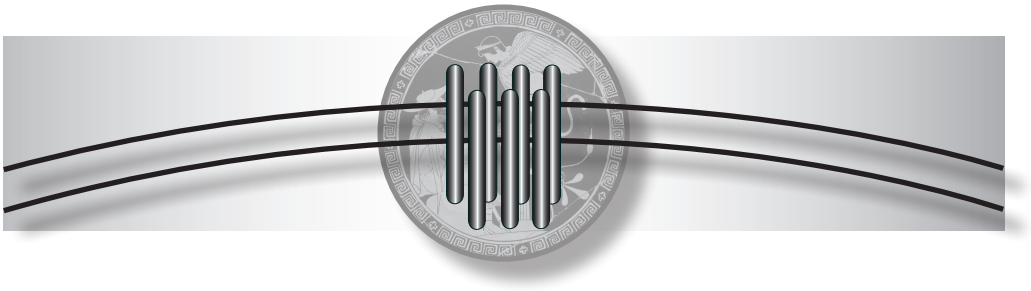
Oo ML, Thangada S, Wu MT, Liu CH, Macdonald TL, Lynch KR, Lin CY, Hla T (2007). Immunosuppressive and anti-angiogenic sphingosine 1-phosphate receptor-1 agonists induce ubiquitinylation and proteasomal degradation of the receptor. *J Biol Chem* **282**:9082-9089

Rhee MH, Nevo I, Levy R, Vogel Z (2000). Role of the highly conserved Asp-Arg-Tyr motif in signal transduction of the CB2 cannabinoid receptor. *FEBS Lett* **466**:300-304

Rovati GE, Capra V, Neubig RR (2007). The highly conserved DRY motif of class A G protein-coupled receptors: beyond the ground state. *Mol Pharmacol* **71**:959-964

Windh RT, Lee MJ, Hla T, An S, Barr AJ, Manning DR (1999). Differential coupling of the sphingosine 1-phosphate receptors Edg-1, Edg-3, and H218/Edg-5 to the G(i), G(q), and G(12) families of heterotrimeric G proteins. *J Biol Chem* **274**:27351-27358

Wong SK (2003). G protein selectivity is regulated by multiple intracellular regions of GPCRs. *Neurosignals* **12**:1-12



Differential response patterns of several ligands at the sphingosine-1-phosphate receptor subtype 3

Chapter 7

Differential response patterns of several ligands at the sphingosine-1-phosphate receptor subtype 3

Manuscript in preparation

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Abstract

Recently, several new ligands for the sphingosine-1-phosphate (S1P) receptor subtype 3 have become available (VPC23019, VPC23153, VPC24191 and the active metabolite of FTY720). The reported potencies and efficacies of these ligands were often based on one read-out system only, but the $S1P_3$ receptor is known to activate multiple signaling pathways. Therefore, we have compared the potencies end efficacies of these ligands in three different assays of $S1P_3$ receptor function (inhibition of cAMP accumulation, elevation of intracellular calcium and receptor internalization).

Our study indicates that the potency and/or efficacy of compounds can vary between assays and that for some compounds the potency can even vary within a particular assay at different stimulation times. Most strikingly, VPC23019 which has been described as an $S1P_{1,3}$ antagonist, behaved as a partial agonist in all three assays. In addition, the potency and efficacy in increasing $[Ca^{2+}]_i$ compared to inhibiting cAMP accumulation or inducing $S1P_3$ internalization was lower for most compounds. Furthermore beside activating G_i-coupled pathways S1P, in contrast to FTY720-P, seemed to additionally activate G_s-coupled pathways in high concentrations.

Based upon our findings we conclude that the $S1P_3$ receptor ligands tested in this study exhibited somewhat different cellular response patterns. An explanation for the observed differences could be that some functional responses require a higher efficacy than others. Alternatively, our observations could point into the direction of ligand-directed signaling for the $S1P_3$ receptor, but definitive proof of the latter awaits further studies.

Introduction

The sphingosine-1-phosphate (S1P) receptors, which bind the bioactive lipid S1P, represent a family of five proteins (named $S1P_1$ to $S1P_5$) which all belong to the family of G-protein coupled receptors (GPCRs) (Chun et al., 2002). One of the S1P receptor subtypes, the $S1P_3$ receptor, is known to play a critical role in the control of cardiac rhythm (Forrest et al., 2004; Sanna et al., 2004) and lung epithelial barrier function (Gon et al., 2005). Interestingly, the role of the $S1P_3$ receptor in cardiac rhythm was recognized due to the cardiac side-effects of FTY720 (Sanna et al., 2004), a S1P agonist which is momentarily in clinical trials for the treatment of multiple sclerosis. Studies using $S1P_3$ knock-out mice, subsequently, suggested that the negative chronotropic effect of FTY720 was predominantly related to the $S1P_3$ receptor expressed in atrial myocytes and on the sino-atrial node (Forrest et al., 2004; Sanna et al., 2004). Recently, it has also been shown that the cardioprotective effects of high density lipoproteins are mediated via $S1P_3$ receptors (Theilmeier, 2006). Furthermore, the anti-atheroslerotic action of FTY720 in ApoE-/- is also believed to be mediated for a major part by $S1P_3$ receptors (Keul et al., 2007).

In lung, $S1P_3$ is exclusively expressed in pulmonary epithelium and activation of $S1P_3$ results in degradation of ZO-1 and claudin leading to a decreased epithelial tight junction formation (Gon et al., 2005). This will lead to the development of paracellular gaps and pulmonary edema. Based upon these data $S1P_3$ receptors may respresent potential new targets in cardiac diseases and disease states associated with a disregulated epithelial barrier function as e.g. Adult Respiratory Distress Syndrome.

In recent years, several new S1P receptor ligands with some specificity for the $S1P_3$ receptor have become commercially available which could be useful tools to further elucidate the physiological role of $S1P_3$ receptors in e.g. the cardiovascular system (Davis et al., 2005). Of these ligands VPC23019, was characterized as a $S1P_{1/3}$ receptor antagonist whereas VPC23153 and VPC24191 were described to have $S1P_{1/3}$ agonistic activity. Although $S1P_3$ receptors are known to activate G_i as well as G_q and $G_{12/13}$-related signal transduction pathways (Young et al., 2006), the pharmacological characterization of these compounds was based on a single functional read-out system (^{35}S-GTPγS binding) only (Davis et al., 2005). However, it is generally known, and has e.g. extensively been studied for the $S1P_5$ receptor (Niedernerg et al., 2003), that the choice of the functional assay system can have implications for the observed efficacy and/or potency of a ligand. In this study

we, therefore, determined the pharmacological properties of the mentioned VPC ligands and the active metabolite of FTY720 on different $S1P_3$-receptor mediated functional responses in transfected CHO-FlpIn cells expressing the human $S1P_3$ receptor.

Materials & Methods

Materials

pcDNA3.1 containing the entire coding region of the human $S1P_3$ receptor was purchased from UMR cDNA Resource Center (Rolla, MO, USA). Cell culture media, hygromycine B, Lipofectamine™ 2000, Optimem, Hank's Balanced Salt Solution (HBSS), pOG44, pcDNA3.1/HisA, pcDNA5/FRT/TO and CHO-FlpIn cells, were obtained from Invitrogen (Breda, The Netherlands). Restriction enzymes (BamHI, XhoI and HindIII) were obtained from Fermentas Life Sciences (St. Leon-Rot, Germany). Pluronic acid and Fluo-4 AM were obtained from Molecular Probes (via Invitrogen). Foetal calf serum (FCS), enzyme free cell dissociation buffer and penicillin/streptomycin were obtained from Gibco (via Invitrogen). LANCE™ cAMP 384 kit and white 384 well optiplates were obtained from Perkin Elmer (Zaventem, Belgium). S1P, (R)-Phosphoric acid mono-[2-amino-2-(6-octyl-1H-benzoimiazol-2-yl) ethyl] ester (VPC23153), (R)-Phosphoric acid mono-[2-amino-2-(3-octyl-phenylcarbamoyl)-ethyl] ester (VPC23019) and (S)-Phosphoric acid mono-[2-amino-3-(4-octyl-phenylamino)-propyl] ester (VPC24191) were obtained from Avanti-Polar Lipids (via Instruchemie B.V., Delfzijl, The Netherlands). Probenecid, EGTA, Triton X-100, 3-isobutyl-1-methylxanthine, pertussis toxin (PTX), bovine serum albumin (BSA) (fatty acid free) and activated charcoal were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands). Black, clear bottom 96 well plates were obtained from Greiner Bio One (Alphen aan den Rijn, The Netherlands). 2-Amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol mono(dihydrogen phosphate) ester (FTY720-P) was synthesized according to previously described methods (Albert et al., 2005).

Molecular cloning, transfection & cell culture

A N-terminal HisG-tag was added to the $S1P_3$ receptor via cloning into pcDNA3.1/HisA using BamHI&XhoI. A second cloning step was done using HindIII&XhoI to clone the HisG-tagged S1P receptor into the expression vector pcDNA5/FRT/TO. CHO-FlpIn cells stably expressing HisG-tagged

$S1P_3$ receptors were constructed and cultured as described before (Chapter 2). The HisG-tag has no influence on signal transduction induced via this receptor (data not shown). The cDNA of the $S1P_3$ receptor obtained from Guthrie contained a point mutation ($G^{962}A$ which results in $R^{321}Q$) which was removed by PCR using the following primers (forward: TGCCTGGTCAGGGGGCGGGGGGCCCG, reverse: CGGGCCCCCCGCCCCCTGACCAGGCA). The $S1P_3$ receptor coding DNA in the resulting plasmid as well as the stable cell line constructed with this plasmid have been confirmed by sequencing.

Calcium- and cAMP assay

Intracellular calcium concentrations ($[Ca^{2+}]_i$) and cAMP levels were measured as described previously (Chapter 2). Stimulations for cAMP measurements were done for 60 minutes unless otherwise indicated. In some cases, S1P elevated forskolin-induced cAMP levels after an initial decrease. For calculations of potency, the curve fit was based on the data points up to those S1P concentrations. To determine the efficacy, the E_{max} of S1P was set to 100 % and the E_{max} of the other compounds was expressed relative to this.

Immunocytochemistry

Immunocytochemistry was performed as described previously (Chapter 3). In short, after overnight serum starvation, cells were stimulated with the indicated ligand in serum free medium for 30 minutes at 37 °C. The N-terminal HisG-tag added to the $S1P_3$ receptor is detected using a 1st anti-HisG antibody combined with AlexaFluor® 488 goat-anti-mouse 2nd fluorescent antibody. The fluorescent signal measured on a microplate reader indicates the amount of receptors on the membrane.

Data analysis

Concentration response curves were analyzed by fitting sigmoidal functions to the experimental data using Prism 4 (Graphpad Software, Inc., San Diego, CA, USA). Data are expressed as means ± s.e.m. One-way ANOVA with a Dunnett's correction or Student's t-test was applied where appropriate. $P<0.05$ was considered significant.

Results

Ligand-induced inhibition of forskolin-induced cAMP accumulation

S1P inhibited forskolin-induced cAMP accumulation in CHO-FlpIn cells expressing the HisG-S1P$_3$ receptor (pEC$_{50}$ = 9.0 ± 0.1, n=14) (Figure 1A, Table 1). FTY720-P had a significantly lower potency to inhibit cAMP accumulation in these cells (pEC$_{50}$ = 8.6 ± 0.1, n=11) (Figure 1A, Table 1). VPC23019, VPC23153 and VPC24191 inhibited forskolin-induced cAMP accumulation, but also with a lower potency than S1P (Table 1). While the efficacy of FTY720-P was similar to S1P, all three VPC compounds showed a significantly lower efficacy compared to S1P and FTY720-P, i.e. behaved as partial agonists (Table 1).

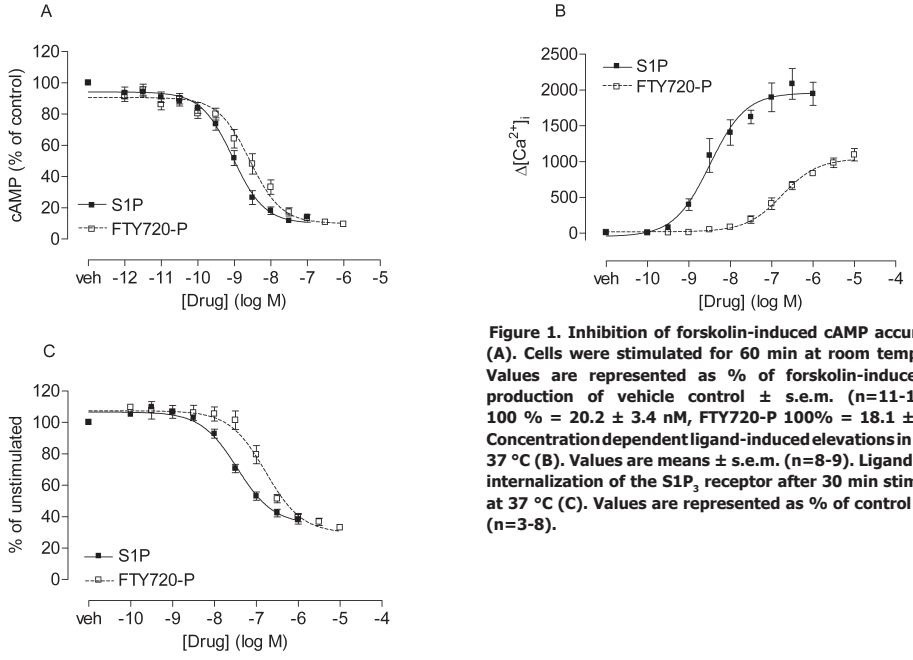


Figure 1. Inhibition of forskolin-induced cAMP accumulation (A). Cells were stimulated for 60 min at room temperature. Values are represented as % of forskolin-induced cAMP production of vehicle control ± s.e.m. (n=11-14). S1P 100 % = 20.2 ± 3.4 nM, FTY720-P 100% = 18.1 ± 2.2 nM. Concentration dependent ligand-induced elevations in [Ca^{2+}]$_i$ at 37 °C (B). Values are means ± s.e.m. (n=8-9). Ligand-induced internalization of the S1P$_3$ receptor after 30 min stimulations at 37 °C (C). Values are represented as % of control ± s.e.m. (n=3-8).

Ligand-induced elevation of [Ca^{2+}]$_i$

S1P increased [Ca^{2+}]$_i$ in these cells (pEC$_{50}$ = 8.4 ± 0.1 (n=9)) (Figure 1B, Table 1). FTY720-P also increased [Ca^{2+}]$_i$ but with a significantly lower potency (pEC$_{50}$ = 6.8 ± 0.1 (n=13)) (Figure 1B, Table 1) and an efficacy of only 51 ± 4 % of that of S1P (Fgure 1B, Table 1). VPC23019, VPC23153 and VPC24191 all raised [Ca^{2+}]$_i$ with significantly lower potencies and efficacies compared to S1P (Table 1).

Ligand-induced receptor internalization

All tested compounds induced internalization of the HisG-S1P$_3$ receptor after 30 min stimulations (for S1P and FTY720-P see figure 1C, for all compounds see Table 1). The potency of FTY720-P as well as all three VPC compounds to induce internalization was significantly lower than of S1P (Table 1). Only VPC23153 and VPC23019 had a lower efficacy compared to S1P (Table 1), whereas VPC24191 and FTY720-P had an efficacy comparable to that of S1P (Table 1).

Table 1. Potency and efficacy values for various ligands on the S1P$_3$ receptor for inhibition of forskolin-induced cAMP accumulation, induced elevations of $[Ca^{2+}]_i$ and induced internalization. The efficacy of S1P in each assay was arbitrarily set at 100 % (cAMP assay 100 % = 90 % inhibition of cAMP accumulation, calcium assay 100 % = 1947 nM and internalization assay 100 % = 60 % decrease compared to unstimulated cells. *p<0.05 compared to S1P

	cAMP			$[Ca^{2+}]_i$			Internalization		
Ligand	pEC_{50}	Efficacy (%)	n	pEC_{50}	Efficacy (%)	n	pEC_{50}	Efficacy (%)	n
S1P	9.0 ± 0.1	100 ± 2	14	8.4 ± 0.1	100 ± 8	9	7.5 ± 0.1	100 ± 4	8
FTY720-P	8.6 ± 0.1*	93 ± 2	11	6.8 ± 0.1*	51 ± 4*	13	6.8 ± 0.1*	109 ± 6	7
VPC23019	6.5 ± 0.1*	79 ± 6*	8	6.1 ± 0.1*	48 ± 4 *	5	5.7 ± 0.2*	72 ± 10*	4
VPC23153	6.2 ± 0.1*	77 ± 7*	9	5.9 ± 0.1*	25 ± 2 *	6	5.6 ± 0.4*	52 ± 18*	4
VPC24191	6.6 ± 0.1*	67 ± 8*	7	5.9 ± 0.1*	56 ± 10*	6	6.0 ± 0.3*	88 ± 5	4

Effects of various incubation times on forskolin-induced cAMP accumulation

To investigate whether the observed differences between ligand efficacies and potencies in both assays were a result of differences in stimulation times (60 min for cAMP and almost instantly for $[Ca^{2+}]_i$), shorter stimulation times were evaluated in the cAMP assay. The potency of S1P to inhibit forskolin-induced cAMP accumulation was increased when instead of 60 min, shorter stimulation times were

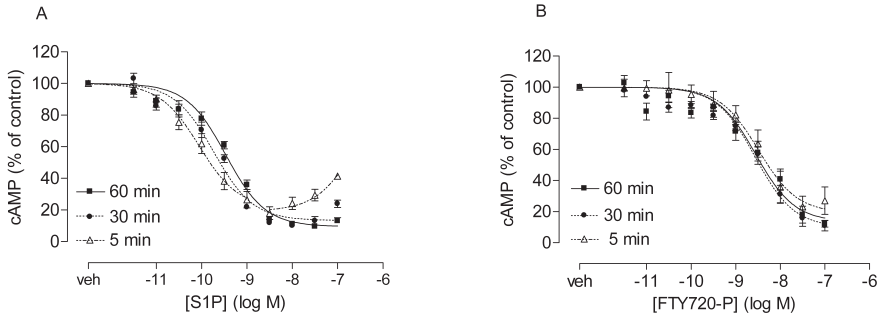


Figure 2. Inhibition of forskolin-induced cAMP accumulation at various stimulation times with S1P (A) or FTY720-P (B). Values are represented as % of forskolin-induced cAMP production of vehicle control ± s.e.m. (n=4).

used (Figure 2A, Table 2). Interestingly, at the shorter stimulation times after an initial decrease an increase in forskolin-induced cAMP accumulation was observed at high concentrations of S1P (> 10 nM).

In contrast to S1P the potency of FTY720-P was not changed at shorter stimulation times (Figure 2B, Table 2). However, all three VPC compounds also had an increased potency at shorter stimulation times (Table 2).

The efficacy of FTY720-P at shorter time points did not change, thus FTY720-P remains a full agonist at the S1P$_3$ receptor at shorter time points.

Table 2. Potencies for S1P, FTY720-P, VPC23019, VPC23153 and VPC24191 at the S1P$_3$ receptor for inhibition of forskolin-induced cAMP accumulation during various stimulation times. Efficacy values were not altered when using different stimulation times (n=4). *p<0.05 compared to 60 min.

	S1P	FTY720-P	VPC23153	VPC23019	VPC24191
Stimulation time	pEC50	pEC50	pEC50	pEC50	pEC50
5 min	10.1 ± 0.1*	8.4 ± 0.1	6.3 ± 0.1*	7.2 ± 0.0*	7.2 ± 0.0*
15 min	9.8 ± 0.1*	8.3 ± 0.2	-	-	-
30 min	9.7 ± 0.1*	8.4 ± 0.1	-	-	-
45 min	9.3 ± 0.1	8.3 ± 0.1	-	-	-
60 min	9.4 ± 0.1	8.3 ± 0.1	5.8 ± 0.2	6.6 ± 0.1	6.7 ± 0.1

Effect PTX on S1P and FTY720-P-induced inhibition of forskolin-induced cAMP accumulation

The observed increase in cAMP levels at higher S1P concentrations could be due to G_s-activation. To reveal the full effect of possible G_s-activation, instead of a combination between G_i and G_s-mediated effects, cells were incubated with PTX to block G_i-activation. Overnight incubation with PTX (100 ng/ml) abolished the inhibitory effect of S1P on forskolin-induced cAMP formation in these cells (data not shown). Additionally, in the PTX treated cells, S1P concentration-dependently stimulated cAMP accumulation, but not in mock transfected cells (Figure 3A). The S1P-induced cAMP accumulation after PTX treatment was further increased upon shorter incubation times with S1P. Stimulation with FTY720-P after PTX treatment did not stimulate cAMP accumulation in these cells (Figure 3B).

Correlation between efficacies of $S1P_3$ receptor ligands in various assays

The efficacies of the tested ligands in the cAMP assay and receptor internalization assay were very similar (Figure 4C, Table 1). However, the efficacies of the tested ligands for inducing elevations of $[Ca^{2+}]_i$ were generally lower than in the cAMP assay and receptor internalization (Figure 4A,B, Table 1).

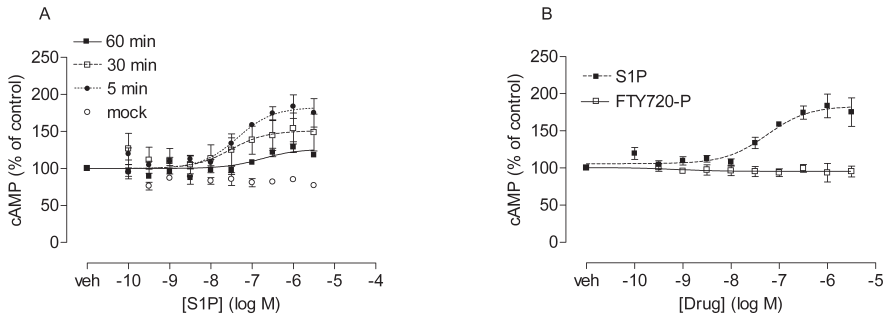


Figure 3. Effect of overnight PTX (100 ng/ml) incubation on S1P-induced changes in forskolin-induced cAMP accumulation at various stimulation times (A) and FTY720-P or S1P-induced changes in forskolin-induced cAMP accumulation at 5 min stimulation (B). The S1P curve in (A) is the same as in (B). Values are represented as % of forskolin-induced cAMP production of vehicle control ± s.e.m. (n=3-4).

Discussion

Recently, several new S1P ligands with some specificity for the $S1P_3$ receptor have become commercially available but they have not been intensively characterized yet. In this study we, therefore, investigated the pharmacological properties of these ligands using different signal transduction assays. Specifically, we determined the potency and efficacy of all ligands with regard to their ability to inhibit forskolin-induced cAMP accumulation, to increase $[Ca^{2+}]_i$ and to internalize the $S1P_3$ receptor In CHO-FlpIn cells expressing the human $S1P_3$ receptor.

In our experiments, both S1P and FTY720-P inhibited forskolin-induced cAMP accumulation, increased $[Ca^{2+}]_i$ and induced $S1P_3$ receptor internalization.

All these responses were mimicked by VPC23019, VPC23153 and VPC24191 (Table 1). In our assay system, VPC23019 thus behaved as a partial agonist rather than an antagonist. Although this may seem strange, differences in efficacy between varying cellular responses (Niedernberg et al., 2003) and/or varying cellular systems have been described before for other GPCRs and can e.g. be caused by differences in receptor expression levels (Sato et al., 2007).

Comparing the potency of the different VPC compounds tested in the cAMP accumulation versus the calcium assay revealed no obvious differences although the potency determined in the cAMP assay consistently tended to be higher than in the calcium assay. This tendency in difference in potency between the cAMP and calcium assay was also observed for S1P and was even more pronounced for FTY720-P. Consequently, the potency of S1P and FTY720-P to inhibit forskolin-induced cAMP accumulation differs only by 0.4 log units, while their potency to increase $[Ca^{2+}]_i$ differed by 1.6 log units (Table 1). For all compounds the potency to induce S1P$_3$ internalization most closely resembled the potency determined in the calcium assay.

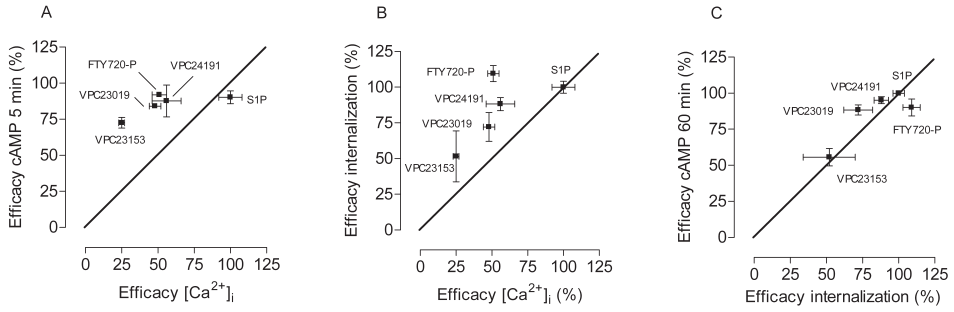


Figure 4. Comparison of efficacy between inhibition of forskolin-induced cAMP accumulation, elevation of $[Ca^{2+}]_i$ and induction of internalization for all used S1P receptor ligands. Efficacies of the 5 min ligand stimulation times for the cAMP assay were compared to the efficacies in the calcium assay, while the 60 ligand stimulation times for the cAMP assay were compared to the internalization assay, since these combinations mostly represent similar assay conditions. The angled lines represent a line of identity rather than correlation lines.

To exclude that the observed differences in potency between the assays are the result of methodological differences (e.g. elevations in $[Ca^{2+}]_i$ are measured instantly upon ligand addition, while effects on cAMP accumulation are measured after 60 min stimulation) the effect of reducing the stimulation time in the cAMP accumulation assay was investigated. Interestingly, the potency of S1P to inhibit forskolin-induced cAMP accumulation was significantly increased upon shorter stimulation times. In contrast, the potency for FTY720-P was not altered with time. Thus, when comparing the potency of S1P and FTY720-P at shorter incubation times, the difference in potency between these compounds in inducing $[Ca^{2+}]_i$ elevations and inhibiting cAMP accumulation was comparable (approximately 1.5 log unit in each assay). In addition, all VPC compounds also showed an increase in potency upon shorter incubation times (Table 2).

Momentarily it is not clear what causes the shift in potency of S1P and/or the VPC compounds in the cAMP assay in time. Probably, it is due to a 'disappearance' of these ligands from the medium, making them less available to the receptor (see also General discussion). Whether this is caused by enzymatic break-down of these ligands, a possible uptake of these ligands or other processes is yet unknown. From these data it can thus be concluded that care should be taken when comparing potencies of ligands using different signal transduction assays especially when different incubation times are used. Because the potency determined in the cAMP assay at the shorter incubation times increased for the majority of compounds, all ligands under these conditions more potently induced changes in cAMP accumulation than in $[Ca^{2+}]_i$.

Another remarkable effect was observed when using shorter S1P stimulation times in the cAMP accumulation assay. We noticed that at these shorter incubation times the initial decrease in forskolin-induced cAMP accumulation was at high concentrations of S1P (>10 nM) followed by an increase in cAMP accumulation. In contrast to S1P, FTY720-P did not increase cAMP accumulation at higher concentrations. Because, CHO cells have been reported to endogenously express S1P receptors (Holdsworth et al., 2005), the observed S1P effect could be due to stimulation of endogenous S1P receptors. However, as the effect was not observed in mock transfected cells, an effect via endogenously expressed receptors is unlikely. The S1P-induced increase in cAMP accumulation was even more pronounced after PTX incubation indicating that in this system S1P activates not only G_i-coupled but also G_s-coupled pathways leading to an increase in cAMP accumulation via stimulation of adenylyl cyclase. Similar observations have been described for the M_2 acetylcholine receptors but only at high receptor density (Michal et al., 2001). In their paper the authors propose that in high receptor expression systems G_i -protein activation gets saturated at higher ligand concentrations resulting in the activation of G_s-proteins by the additionally activated receptors.

Another explanation for the effects we observed in this study could be, that the rise in cAMP accumulation is caused by a G_q-mediated response via calcium-dependent adenylyl cyclases. Based upon our data, this cannot be excluded. Although FTY720-P did not have stimulatory effects on cAMP accumulation the efficacy of this compound to induce elevations of $[Ca^{2+}]_i$ is also much lower than that of S1P. Compared to S1P, FTY720-P is a partial agonist in the calcium assay whereas it behaves as a full agonist at all stimulation times in the cAMP assay.

Because FTY720-P does not activate $S1P_2$ receptors, the observed difference in efficacy of S1P versus FTY720-P in the calcium assay could still be explained by a synergistic functioning of the $S1P_3$ receptor and an endogenous $S1P_2$ receptor. In mock transfected cells, S1P induced small increases in $[Ca^{2+}]_i$, whereas FTY720-P had no effect, which also argues in favour of S1P partly acting via an endogenously expressed receptor (Chapter 6). In addition to FTY720-P, there is also quite a difference in efficacy for VPC23153 and VPC23019 between the two assays (Table 1). It can thus be concluded that the majority of compounds is more efficacious in inhibiting forskolin-induced cAMP accumulation than in increasing $[Ca^{2+}]_i$. In addition, there is a striking similarity between the efficacies to induce internalization and the efficacies to inhibit cAMP accumulation. Therefore most likely, a higher efficacy is required for calcium signaling compared to inhibition of cAMP accumulation and/or internalization.

However, an alternative explanation based on a relatively new concept in GPCR pharmacology could be that some of the tested compounds differentially activate the signal transduction pathways investigated. Classical pharmacology assumed that all ligands activating a given GPCR yield similar responses on the same signal transduction pathways which only differ with regard to agonist potency and efficacy. However, recent evidence from several GPCRs demonstrates that some agonists preferentially elicit a certain response whereas others, acting on the same receptor, preferentially elicit another response, and that such differences cannot be explained in terms of agonist potency or efficacy (for an overview see Urban et al., 2007). These observations have led to the new concept that each agonist can induce a different conformational state of the receptor which results in preferential coupling to specific G-proteins and subsequent activation of specific signal transduction pathways. This phenomenon has been described in many different systems, and has been termed in as many different ways, including ligand-directed signaling, protean agonism, functional selectivity, conformational selection or ligand-directed trafficking (Simmons, 2005; Urban et al., 2007).

Interestingly, for the $S1P_1$ receptor, some observations suggesting that different ligands induce different receptor conformations have already been described. $S1P_1$ receptors internalized upon S1P or SEW2871 stimulation and were eventually recycled to the plasma membrane, while internalized upon stimulation with the immunosuppressive prodrug FTY720 these receptors were broken down in lysozomes (Oo et al., 2007). This difference in receptor fate has been related to

polyubiquitinylation of the $S1P_1$ receptor induced by FTY720-P, but not by S1P (Oo et al., 2007). In Chapter 6 we also show some data suggesting conformational differences between S1P and FTY720-P as a particular mutation in the second intracellular loop of the $S1P_1$ receptor did affect S1P but not FTY720-induced G_i-signaling. Although ligand-directed signaling may explain some of the effects observed in our study further investigation is necessary before drawing any definite conclusions. In addition, as neither the order of potency nor the order of efficacy of the tested ligands was markedly different between the assays, strong indications for ligand-directed signaling are missing.

Overall it is clear that several of the used S1P receptor ligands show some distinctions in $S1P_3$-receptor mediated responses. The majority of ligands had lower potencies and efficacies in increasing $[Ca^{2+}]_i$ compared to inhibiting cAMP accumulation. Furthermore S1P, in contrast to FTY720-P, seemed to additionally activate the G_s-pathway. Although these results may point in the direction of ligand-directed signaling definite proof to confirm this is still missing.

References

Albert R, Hinterding K, Brinkmann V, Guerini D, Muller-Hartwieg C, Knecht H, Simeon C, Streiff M, Wagner T, Welzenbach K, Zecri F, Zollinger M, Cooke N, Francotte E (2005). Novel immunomodulator FTY720 is phosphorylated in rats and humans to form a single stereoisomer. Identification, chemical proof, and biological characterization of the biologically active species and its enantiomer. *J Med Chem* **48**:5373-5377

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

Davis MD, Clemens JJ, Macdonald TL, Lynch KR (2005). Sphingosine 1-phosphate analogs as receptor antagonists. *J Biol Chem* **280**:9833-9841

Forrest M, Sun SY, Hajdu R, Bergstrom J, Card D, Doherty G, Hale J, Keohane C, Meyers C, Milligan J, Mills S, Nomura N, Rosen H, Rosenbach M, Shei GJ, Singer II, Tian M, West S, White V, Xie J, Proia RL, Mandala S (2004). Immune cell regulation and cardiovascular effects of sphingosine 1-phosphate receptor agonists in rodents are mediated via distinct receptor subtypes. *J Pharmacol Exp Ther* **309**:758-768

Gon Y, Wood MR, Kiosses WB, Jo E, Sanna MG, Chun J, Rosen H (2005). S1P3 receptor-induced reorganization of epithelial tight junctions compromises lung barrier integrity and is potentiated by TNF. *Proc Natl Acad Sci U S A* **102**:9270-9275.

Holdsworth G, Slocombe P, Hutchinson G, Milligan G (2005). Analysis of endogenous S1P and LPA receptor expression in CHO-K1 cells. *Gene* **350**:59-63

Im DS, Heise CE, Ancellin N, O'Dowd BF, Shei GJ, Heavens RP, Rigby MR, Hla T, Mandala S, McAllister G, George SR, Lynch KR (2000). Characterization of a novel sphingosine 1-phosphate receptor, Edg-8. *J Biol Chem* **275**:14281-14286

Keul P, Sattler K, Levkau B (2007). HDL and its sphingosine-1-phosphate content in cardioprotection. *Heart Fail Rev* **12**:301-306

Michal P, Lysíková M, Tucek S (2001). Dual effects of muscarinic M(2) acetylcholine receptors on the synthesis of cyclic AMP in CHO cells: dependence on time, receptor density and receptor agonists. *Br J Pharmacol* **132**:1217-1228

Niedernberg A, Tunaru S, Blaukat A, Harris B, Kostenis E (2003). Comparative analysis of functional assays for characterization of agonist ligands at G protein-coupled receptors. *J Biomol Screen* **8**:500-510.

Oo ML, Thangada S, Wu MT, Liu CH, Macdonald TL, Lynch KR, Lin CY, 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

Sanna MG, Liao J, Jo E, Alfonso C, Ahn MY, Peterson MS, Webb B, Lefebvre S, Chun J, Gray N, Rosen H (2004). Sphingosine 1-phosphate (S1P) receptor subtypes S1P1 and S1P3, respectively, regulate lymphocyte recirculation and heart rate. *J Biol Chem* **279**:13839-13848

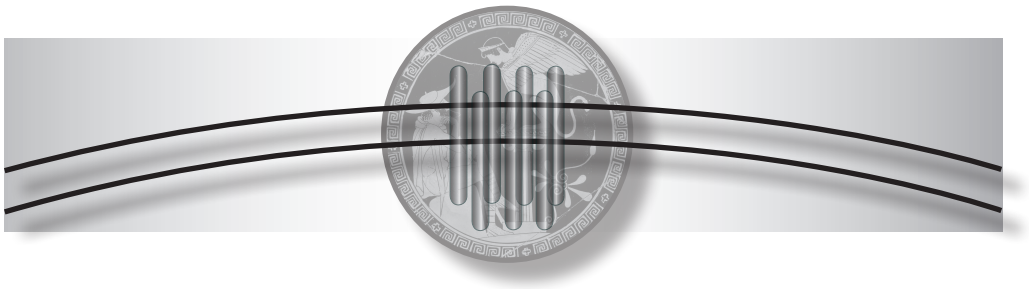
Sato M, Horinouchi T, Hutchinson DS, Evans BA, Summers RJ (2007). Ligand-directed signaling at the β3-adrenoceptor produced by SR59230A relative to receptor agonists. *Mol Pharmacol* in press

Simmons MA (2005). Functional Selectivity, Ligand-Directed Trafficking, Conformation-Specific Agonism: What's In A Name? *Mol Interv* **5**:154-157.

Theilmeier G, Schmidt C, Herrmann J, Keul P, Schäfers M, Herrgott I, Mersmann J, Larmann J, Hermann S, Stypmann J, Schober O, Hildebrand R, Schulz R, Heusch G, Haude M, von Wnuck Lipinski K, Herzog C, Schmitz M, Erbel R, Chun J, Levkau B (2006). High-density lipoproteins and their constituent, sphingosine-1-phosphate, directly protect the heart against ischemia/reperfusion injury in vivo via the S1P3 lysophospholipid receptor. *Circulation* **114**:1403-1409

Urban JD, Clarke WP, von Zastrow M, Nichols DE, Kobilka B, Weinstein H, Javitch JA, Roth BL, Christopoulos A, Sexton PM, Miller KJ, Spedding M, Mailman RB (2007). Functional selectivity and classical concepts of quantitative pharmacology. *J Pharmacol Exp Ther* **320**:1-13

Young N, Van Brocklyn JR (2006). Signal transduction of sphingosine-1-phosphate G protein-coupled receptors. *ScientificWorldJournal* **6**:946-966



General discussion

Chapter 8

General discussion

Sphingosine-1-phosphate (S1P) regulates various cellular processes via a family of five specific G-protein coupled receptors (GPCRs), named $S1P_{1-5}$ receptors (Chun et al., 2002). Being discovered less than two decades ago, many questions and "riddles" about the role and biology of these receptors in health and disease are still unanswered. Because S1P receptors are a relatively newly discovered family of receptors, the tools available to investigate these receptors such as subtype-selective agonists and/or antagonists are very limited. In addition, the available S1P ligands are generally not that well characterized. The first part of this thesis describes the development and/or pharmacological characterization of some tools which should facilitate the investigation of the molecular pharmacology of S1P receptors. In the second part of the thesis the signaling and regulation of S1P receptors is described in cell lines stably expressing the receptor, primary vascular cells or whole blood vessels. As a main focus in our department is the cardiovascular system, these studies have been restricted to the $S1P_1$, $S1P_2$ and $S1P_3$ receptors, as these subtypes are suggested to be the most important S1P receptors in the cardiovascular system (Michel et al., 2007).

Part I: Tools

As already stated above, selective S1P ligands are relatively few, and most of the newly developed ligands are often not that well characterized pharmacologically. For example suramin has been reported to act as an antagonist specific for the $S1P_3$ subtype (Salomone et al., 2003). However, this compound has many other effects on cellular signaling, e.g. suramin has been described as a kind of general inhibitor of GPCR signaling because it inhibits receptor-G-protein coupling (Beindl et al., 1996). In this thesis (Chapter 2), we pharmacologically characterized BML-241, a compound that was suggested to be an interesting lead compound for the development of $S1P_3$ antagonists. In the original publication this compound was found to inhibit

S1P-induced elevations in $[Ca^{2+}]_i$ in cells expressing the $S1P_3$ receptor but not in cells expressing the $S1P_1$ receptor (Koide et al., 2002). However, BML-241 was only tested at one relatively high concentration of 10 μM in that study. One should thus be careful to draw any conclusions based upon these results. For example, elevations of $[Ca^{2+}]_i$ via $S1P_1$ receptor activation have been thought to be a result of the βγ-subunits of G_i-proteins (Okamoto et al., 1998), while in case of the $S1P_3$ receptor, G_q-proteins are involved (Kon et al., 1999). An alternative explanation for the effects observed for BML-241 could thus be that BML-241 behaves as a G_q-inhibitor instead of a $S1P_3$ receptor inhibitor. In our study we confirmed the inhibitory effect of high BML-241 concentrations on the $S1P_3$ receptor-mediated increases in $[Ca^{2+}]_i$. However, BML-241 also inhibited $S1P_2$-, α_{1A}-adrenergic- and purinergic receptor-mediated effects on $[Ca^{2+}]_i$ and reduced the α_1-adrenergic-mediated contraction of rat mesenteric artery (Chapter 2). The effects observed on α_{1A}-adrenergic receptor signaling were not the result of a direct interaction of BML-241 with this receptor as, based upon competition binding studies, BML-241 lacked high affinity for this receptor. Perhaps even more importantly, BML-241 did not antagonize the $S1P_3$ receptor-mediated inhibition of forskolin-induced cAMP accumulation, demonstrating that it does not inhibit the receptor itself but rather certain signaling responses which can be elicited by the $S1P_3$ receptor. From our study we thus conclude that BML-241 indeed inhibits $S1P_3$ mediated effects on $[Ca^{2+}]_i$ but that it is most likely a low potency, non-selective inhibitor of increases in $[Ca^{2+}]_i$, rather than a specific antagonist at the $S1P_3$ receptor.

In our own search for specific S1P receptor ligands, we have performed some medium throughput screening. These studies were done in close collaboration with Dr. I. de Esch from the department of Medicinal Chemistry from the Free University (Amsterdam) and Dr. J. Brussee and Dr. M.W. Beukers from the division of Medicinal Chemistry from Leiden University (Leiden). In this screening approach two different strategies to select a series of compounds were used. One strategy was based on a pharmacophore model for the $S1P_1$ receptor whereas the other was a selection of compounds with high structural diversity. The first strategy identified a $S1P_1$ selective lead compound whereas in the second strategy a $S1P_3$-selective lead compound was identified. Based upon the lead compounds two new series of ligands with close homology to the lead compounds were selected to determine some structure activity relationships for these compounds. For confidentiality reasons the screening efforts and results are no part of this thesis but are promising

for further development of selective $S1P_1$ and/or $S1P_3$ ligands.

Another important tool in research, besides the ligands, is the existence or development of innovative methods to study GPCR signaling. In this respect, Hoffmann et al. (2005) have published an elegant method using a fluorescein arsenical hairpin binder (FlAsH)-based fluorescence resonance energy transfer (FRET) approach to study the activation-induced conformational changes in the adenosine A_{2A} receptor. In their studies a FlAsH-motif of six amino acids was inserted into the third intracellular loop of the receptor, and a cyan fluorescent protein (CFP) was attached to the C-terminal tail of the receptor. When the CFP and the FlAsH are in close proximity, CFP can act as a fluorescent donor and thereby transfer energy to the acceptor (FlAsH). Upon activation, a conformational change is induced in the receptor which may subsequently change the orientation and distance between the donor and acceptor thus altering the energy transfer between these two. In collaboration with Dr. C. Hoffmann from the Institute of Pharmacology and Toxicology of the University of Würzburg (Würzburg, Germany) we applied the FlAsH-based FRET approach to the $S1P_1$-receptor. Usually, to allow the best signal to occur, the receptor constructs need to be optimized for the relative distance and orientation of the two flourophores. This procedure aims to place the two fluorophores within the Föster-distance and thus allowing FRET to occur. Since structural data are only available for rhodopsin, this process is still laborus and needs to be individually adjusted for each receptor.

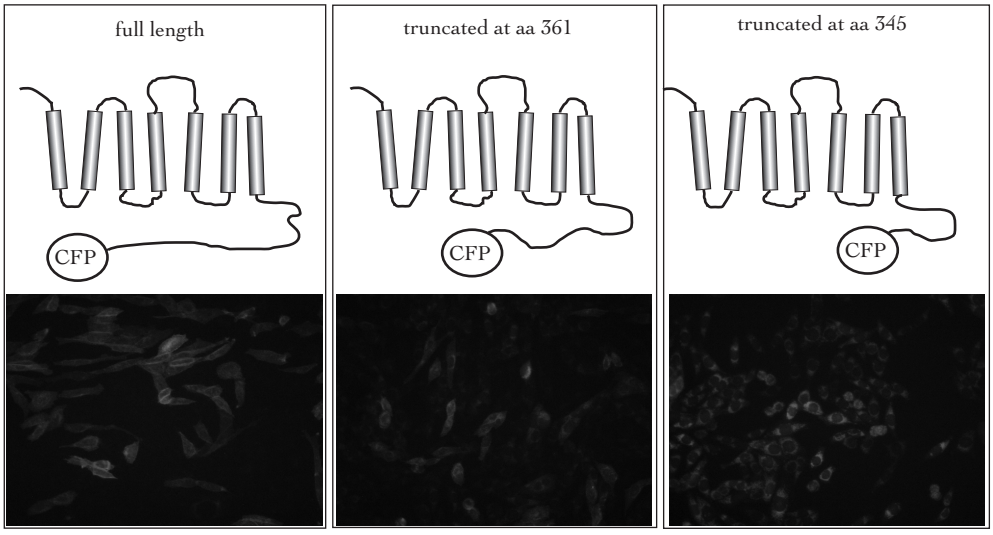


Figure 1. Constructs of CFP-tags added to truncated $S1P_1$ receptor with corresponding fluorescent images CHO-FlpIn cells expressing these constructs.

To do so we used two rounds of optimization. In the first set of experiments a C-terminal CFP tag was added to the $S1P_1$ receptor in three different positions and the membrane expression of the tagged construct was studied. As the orientation of the CFP tag to the FlAsH motif is of major importance, besides the full length construct also two C-terminal truncated $S1P_1$ receptor constructs, either truncated

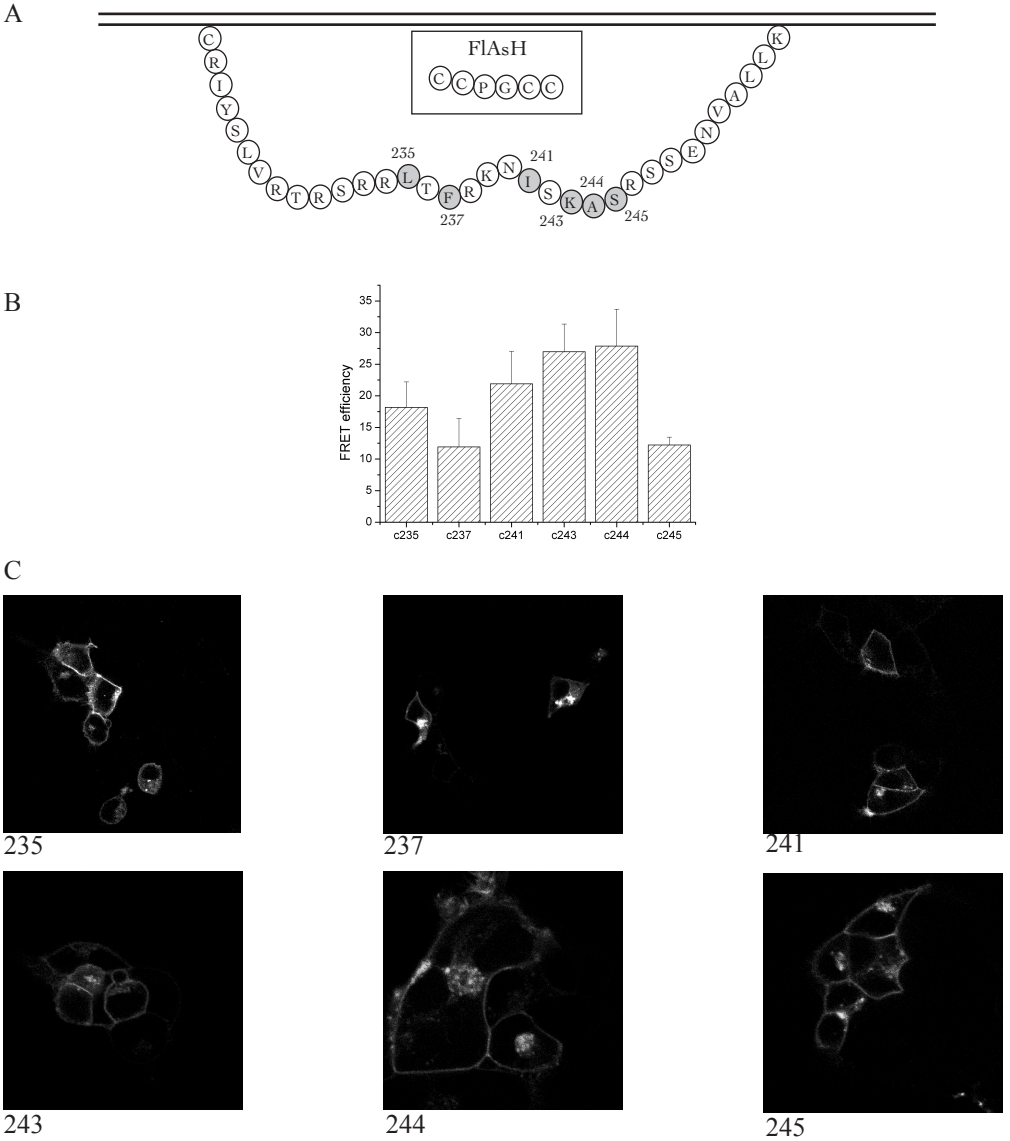


Figure 2. Amino acid sequence of the third intracellular loop of the $S1P_1$ receptor (A). Grey circles represent the amino acid after which the FlAsH construct has been inserted. FRET efficiencies of $S1P_1$ receptor constructs with a truncated C-terminus (at amino acid 361) and inserted FlAsH (numbers correspond to A) (B). FRET efficiency is measured by determining the change in FRET in time after removing the FlAsH ligand. Fluorescent images showing membrane expression of the corresponding $S1P_1$ receptor constructs in CHO-FlpIn cells (C). Receptors are visualized via the C-terminal CFP-tag.

at the amino acid 361 ($S1P_1C^{361}$trunc) or amino acid 345 ($S1P_1C^{345}$trunc) position, have been created and the CFP tag has been added to them (Figure 1). Two of the constructs, the full length and the $S1P_1C^{361}$ truncated receptor showed plasma membrane expression whereas the $S1P_1C^{345}$ truncated receptor was predominantly located in the cytosol (Figure 1). In a second set of experiments the FlAsH motif (CCPGCC) was inserted at amino acid position 239 in the third intracellular loop of the full length and $S1P_1C^{361}$ truncated receptor. The selection of these sites was reasoned to be suitable, since they closly correspond to sites which had been used for the adenosine A_{2A} receptor. The $S1P_1C^{361}$ truncated receptor showed some FRET efficiency, which is measured by removing the FlAsH ligand and subsequently determined the change in FRET in time, but this efficiency was not changed by the addition of S1P (data not shown). Therefore, we made six additional constructs in which the FlAsH motif was inserted at different positions in the third intracellular loop of the $S1P_1C^{361}$ truncated CFP-tagged receptor (Figure 2A). Although all constructs were expressed at the cell membrane (Figure 2C) and they all showed some FRET efficiency (Figure 2B), the FRET ratio was not changed in the presence of S1P (Figure 3), SEW2871, FTY720 or FTY720-P (data not shown). Probably due to the fact that the third intracellular loop of the $S1P_1$ receptor compared to the adenosine A_{2A} receptor is much shorter, it is possibly less flexible. Consequently, the change in position of intracellular loop 3 (with the FlAsH element) compared to the C-terminus (with the CFP) upon activation could be too moderate to result in any changes of the FRET signal. Since these results for the $S1P_1$ receptor were not that promising we did not pursue this project any further. An interesting finding was also that the C-terminus of the $S1P_1$ receptor between amino acid 361 and amino

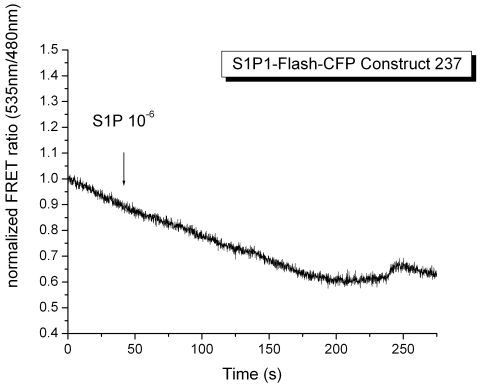


Figure 3. FRET ratio of the $S1P_1$-CFP-237 receptor construct. S1P (1μM) is added at the indicated time and the FRET ratio (535nm/480nm) is followed in time.

acid 345 seem to have an important role in receptor surface expression.
One major advantage of FlAsH, compared to other fluorescent tags as e.g. green fluorescent protein (GFP), is its small size. Fluorescent tags are often used to investigate receptor regulatory processes such as internalization, recycling and down regulation. However, because of the relative large size of these fluorescent tags, they can affect expression, signaling and other receptor-mediated processes (McLean & Milligan, 2000). Since we were interested in investigating regulatory processes of S1P receptors such as internalization, we set up a method that allowed the detection of receptors at the cell surface on a medium throughput scale (96 wells plate) (Chapter 3). This immunocytochemical method was based on the addition of a small N-terminal HisG-tag to the receptor and the subsequent detection of this tag with an anti-HisG antibody and a e.g. fluorescent second antibody. The small N-terminal tag did not affect membrane expression and signaling properties of the receptor. The method was optimized using the $S1P_1$ receptor as a model receptor comparing several first and second antibodies, ranging from absorbance to fluorescent en luminescent read outs. The combination of a specific first antibody for the HisG-tag and a fluorescent second antibody (AlexaFluor® 488) proved most reliable and sensitive. The developed method was suitable to investigate the agonist-induced internalization of the $S1P_1$ receptor but also of other S1P receptors (Chapter 5) and could be performed in a 96 well plate and quantified using a microplate reader.

Part II: S1P receptor signaling and regulation

In the cardiovascular system, several S1P receptors are expressed, most importantly the $S1P_1$, $S1P_2$ and $S1P_3$ receptor. Many things are still unclear about their regulation and signaling. Measuring the effect of exogenously added S1P on vascular preparations in an organ bath set up is difficult due to technical difficulties. However, recently some S1P receptor ligands, selective and non-selective, have become commercially available and enabled a detailed study on the role of S1P receptors in vascular responses. The study described in this thesis (Chapter 4) investigated the effects of some S1P ligands on contraction/relaxation of aortic rings and on S1P receptor-mediated signaling in vascular cells (smooth muscle and endothelial cells) isolated from the aorta.
We were the first to show that all the S1P receptor ligands induce an endothelium-dependent $S1P_1$ and/or $S1P_3$ receptor-mediated relaxation of aortic rings most likely

via an activation of endothelial nitric oxide synthase (eNOS) and the subsequent production of the vasodilator NO. When studying the two main cell types of the aorta separately, all ligands affected the forskolin-induced cAMP accumulation in rat aortic endothelial cells (RAECs). The majority of ligands inhibited cAMP accumulation in these cells, except for VPC23153 which increased the cAMP accumulation. In addition, the $S1P_1$/$S1P_3$ antagonist VPC23019 competitively antagonized the effect of FTY720-P on cAMP accumulation in RAECs. Since both, ligands increasing and decreasing endothelial cAMP, were associated with vasorelaxation, the vasodilation appears to be independent of endothelial cAMP content. Besides the effects on cAMP accumulation, S1P and FTY720-P at high concentrations also moderately elevated $[Ca^{2+}]_i$ in RAECs but it is not known whether these effects are indeed S1P receptor mediated. Because cultured RAECs predominantly show $S1P_1$-receptor mediated signaling these cells most express only the $S1P_1$ receptor, a finding that was supported by qPCR data.

In vascular smooth muscle cells (VSMCs), FTY720, FTY720-P and SEW2871 slightly inhibited forskolin-induced cAMP accumulation, but the inhibitory effect was too small to allow reliable quantification. Of all tested ligands, only the one with $S1P_2$ agonistic activity (S1P) elevated $[Ca^{2+}]_i$ in these cells. In addition, the $S1P_2$ receptor antagonist JTE013 partly inhibited this effect. qPCR showed that the $S1P_1$, $S1P_2$ and $S1P_3$ receptors were expressed in these cells, but apparently signaling in these cells occurs predominantly via the $S1P_2$ receptor. This led to the overall conclusion that in the two main cell types of a vessel, being smooth muscle cells and endothelial cells, different S1P receptors are likely to cause opposite effects on vascular tone. Mainly the $S1P_1$ receptor, but possibly also the $S1P_3$ receptor, in endothelial cells (ECs) induces a relaxant effect upon S1P stimulation, while mostly stimulation of the $S1P_2$ receptor in VSMCs causes a contractile effect. The net effect of S1P ligands in a vessel is, therefore, most likely a result of both effects, depending on receptor expression and or regulation.

As found for other GPCRs, S1P receptors are also subject to regulatory processes. Using the immunocytochemical method we have developed (Chapter 3), the internalization of S1P receptors which are expressed in the cardiovascular system was compared (Chapter 5). Some interesting differences were found between the $S1P_1$ and $S1P_2$ receptor on one hand and the $S1P_3$ receptor on the other hand. The $S1P_3$ receptor internalized faster, required lower concentrations of S1P and was internalized more efficiently. The differences in internalization between the

S1P receptor subtypes could only partly be explained by differences in signaling between these receptors. Pertussis toxin treatment showed the involvement of G_i-protein activation for the $S1P_3$ receptor but not for the $S1P_1$ and $S1P_2$ receptor. However, cAMP signaling itself or calcium signaling was not involved in internalization of each of the three S1P receptors. Since $[Ca^{2+}]_i$ is increased via $S1P_3$ receptor stimulation to a much larger extent than via $S1P_1$ receptor stimulation, we hypothesized that this may play a role in internalization. However, increasing $[Ca^{2+}]_i$ in $S1P_1$ expressing cells or blocking $[Ca^{2+}]_i$ elevations in $S1P_3$ expressing cells did not change the internalization characteristics of either receptor. The observed differences in internalization between the S1P receptors can thus not be completely explained based upon our study. In this respect it is interesting to mention that it has been demonstrated by others that the $S1P_3$ receptor in contrast to the $S1P_1$ receptor, does not require phosphorylation to be internalized (Rutherford et al., 2005). Probably this can explain the fast internalization of the $S1P_3$ compared to the $S1P_1$ and $S1P_2$ receptor.

As mentioned before, the signaling proterties of the $S1P_1$, $S1P_2$ and $S1P_3$ receptor are different. The $S1P_1$ receptor exclusively activates G_i-proteins whereas the $S1P_2$ and $S1P_3$ receptor can activate various different G-proteins. One conserved domain that has been shown to play an important role in G-protein activation of GPCRs is the DRY motif, which is located at the boundary of the third transmembrane domain and the second intracellular loop. Interestingly, the S1P receptors show a remarkable variance in this highly conserved motif. The $S1P_1$ receptor is the only receptor that contains an ERY motif, while the other S1P receptors contain either an ERH, ERF or ERS motif. We therefore investigated whether the tyrosine residue of the D/ERY motif is involved in restricting $S1P_1$ receptor coupling (Chapter 6). Mutating the tyrosine residue to a histidine or alanine residue markedly decreased the potency of S1P to inhibit cAMP accumulation whereas the potency of FTY720-P was unchanged. The effect on the potency of the $S1P_1$ selective agonist SEW2871 mimicked that of S1P, since mutations in the D/ERY motif also decreased the potency of SEW2871. Mutations in the ERH motif of the $S1P_2$ and $S1P_3$ receptor did not induce many changes in signaling, apart from the $S1P_2$ $H^{131}Y$ mutant receptor which showed an enhanced increase in cAMP accumulation upon S1P stimulation. Overall, we conclude that tyrosine residue of the D/ERY motif of the $S1P_1$ receptor does not restrict the receptor from promiscuous coupling or influence constitutive receptor activity as seen for some other GPCRs. However, the mutations somehow

influence the conformational state of the receptor thereby affecting G_i-signaling in response to S1P and SEW2871 but not to FTY720-P. Besides investigating the signaling properties of the mutant receptors we also studied internalization of these receptors in additional experiments. Pilot experiments for the $S1P_1$ receptor mutants indicated that the effects of the mutations observed on G_i-signaling were comparable to the effects found on internalization (data not shown). For the $S1P_2$ and $S1P_3$ mutant receptors no differences in S1P-induced internalization between wild type and mutant receptors were observed (Figure 4). Because the results on

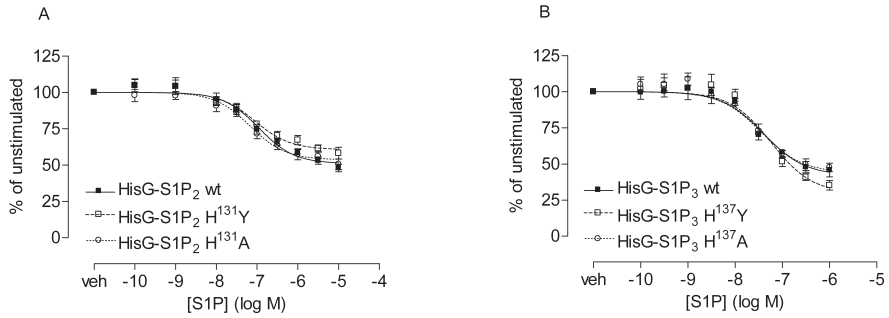


Figure 4. Concentration-dependent S1P-induced internalization of the $S1P_2$ (A) and $S1P_3$ (B) receptor wt and D/ERY-mutants. Stimulations were carried out at 37 ºC for 30 min. Values are expressed as percentage of fluorescent signal measured for unstimulated cells which was set at 100% and are presented as means ± s.e.m. (n=4-13).

internalization were similar to the results obtained for cAMP accumulation for the $S1P_1$ and $S1P_3$ receptor, this could suggest a link between the two pathways.
Recently, several new S1P receptor ligands have become commercially available. In the final part of this thesis, we investigated the signaling of these ligands via the $S1P_3$ receptor (Chapter 7). As this receptor can activate both, G_i and G_q-coupled pathways, the potency and efficacy of a series of compounds on inhibition of the forskolin-induced cAMP production (G_i-mediated) and elevation of $[Ca^{2+}]_i$ (G_q-mediated) was determined. In addition, the potency and efficacy of these compounds to induce $S1P_3$ receptor internalization was investigated. Our study found some interesting differences in the ability of some ligands to activate either G_i or G_q-coupled pathways. For example, the phosphorylated and active form of FTY720, FTY720-P was a full agonist when inhibiting the forskolin-induced cAMP accumulation whereas it had only partial agonistic activity in inducing elevations in $[Ca^{2+}]_i$. Similarly, the $S1P_1$ and $S1P_3$ agonist VPC23153, proved more efficient in inducing G_i-mediated inhibition of cAMP accumulation than in G_q-mediated elevation of $[Ca^{2+}]_i$ via the $S1P_3$ receptor.

In general, the efficacy of the tested ligands to induce $S1P_3$ receptor internalization matched more closely with their efficacy to inhibit cAMP accumulation than with their efficacy to elevate $[Ca^{2+}]_i$. Another interesting finding that could point in the direction of ligand-directed signaling of the $S1P_3$ receptor is the finding that at high concentrations S1P but not FTY720-P induces an increase in cAMP accumulation most likely via the activation of G_s-proteins. Besides our findings suggesting ligand-directed signaling at the $S1P_3$ receptor, it also becomes clear from our study that care should be taken before drawing any definite conclusion about ligand-directed signaling. Most importantly, when comparing potencies determined in signal transduction assays which differ in incubation time it is important to be sure that the ligand concentration is stable in time. Pilot experiments in our laboratory indicated that the concentration of S1P itself declines in time (Figure 5), probably due to degradation and/or uptake, resulting in an underestimation of the potency determined in the cAMP assay when using longer incubation times. In addition, in case the cells used to investigate ligand-directed signaling contain endogenous receptors that also can be activated by some of the ligands under investigations one should also be careful before drawing any conclusions. In conclusion, our study indicates that S1P compared to FTY720-P differentially activates $S1P_3$-mediated cAMP and calcium signaling pathways probably because both ligands induce different conformations of the $S1P_3$ receptor.

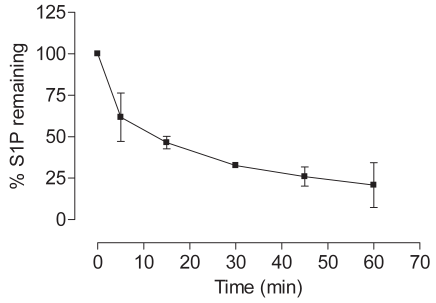


Figure 5. A pilot experiment where disappearance of S1P from stimulation buffer was measured using an HPLC based method (Butter et al., 2005). 2 ml stimulation buffer was incubated with CHO-FlpIn cells expressing the $S1P_3$ receptor for the indicated times at 37 ºC in a 6 wells plate. S1P concentrations were measured using an HPLC method. Data was normalized to the starting S1P concentration as measured by HPLC.

References

Beindl W, Mitterauer T, Hohenegger M, Ijzerman AP, Nanoff C, Freissmuth M (1996). Inhibition of receptor/G protein coupling by suramin analogues. *Mol Pharmacol* **50**:415-423

Butter JJ, Koopmans RP, Michel MC (2005). A rapid and validated HPLC method to quantify sphingosine 1-phosphate in human plasma using solid-phase extraction followed by derivatization with fluorescence detection. *J Chromatogr B Analyt Technol Biomed Life Sci* **824**:65-70

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

Hoffmann C, Gaietta G, Bünemann M, Adams SR, Oberdorff-Maass S, Behr B, Vilardaga JP, Tsien RY, Ellisman MH, Lohse MJ (2005). A FlAsH-based FRET approach to determine G protein-coupled receptor activation in living cells. *Nat Methods* **2**:171-176.

Koide Y, Hasegawa T, Takahashi A, Endo A, Mochizuki N, Nakagawa M, Nishida A (2002). Development of novel EDG3 antagonists using a 3D database search and their structure-activity relationships. *J Med Chem* **45**:4629-4638

Kon J, Sato K, Watanabe T, Tomura H, Kuwabara A, Kimura T, Tamama K, Ishizuka T, Murata N, Kanda T, Kobayashi I, Ohta H, Ui M, Okajima F (1999). Comparison of intrinsic activities of the putative sphingosine 1-phosphate receptor subtypes to regulate several signaling pathways in their cDNA-transfected Chinese hamster ovary cells. *J Biol Chem* **274**:23940-23947

McLean AJ, Milligan G (2000). Ligand regulation of green fluorescent protein-tagged forms of the human β1-and β2-adrenoceptors; comparisons with the unmodified receptors. *Br J Pharmacol* **130**:1825-1832

Michel MC, Mulders AC, Jongsma M, Alewijnse AE, Peters SL (2007). Vascular effects of sphingolipids. *Acta Paediatr Suppl* **96**:44-48

Okamoto H, Takuwa N, Gonda K, Okazaki H, Chang K, Yatomi Y, Shigematsu H, Takuwa Y (1998). EDG1 is a functional sphingosine-1-phosphate receptor that is linked via a Gi/o to multiple signaling pathways, including phospholipase C activation, Ca2+ mobilization, Ras-mitogen-activated protein kinase activation, and adenylate cyclase inhibition. *J Biol Chem* **273**:27104-27110

Rutherford C, Ord-Shrimpton FU, Sands WA, Pediani JD, Benovic JL, McGrath JC, Palmer TM (2005). Phosphorylation-independent internalization and desensitization of the human sphingosine-1-phosphate receptor S1P3. *Cell Signal* **17**:997-1009

Salomone S, Yoshimura S, Reuter U, Foley M, Thomas SS, Moskowitz MA, Waeber C (2003). S1P3 receptors mediate the potent constriction of cerebral arteries by sphingosine-1-phosphate. *Eur J Pharmacol* **469**:125-134

Summary

Sphingosine-1-phosphate (S1P) is a bioactive lipid that regulates many cellular functions. Currently, five G-protein coupled receptors (GPCRs) are known which are specifically activated by S1P, resulting in a wide range of cellular effects.

Chapter 1 of this thesis gives a general introduction into GPCRs with a special focus on S1P receptors. Their signaling and effects on a molecular level are described, together with the regulation of GPCRs upon stimulation. Furthermore, the role of S1P receptors in the cardiovascular system is described. Also, various tools to study receptor signaling are discussed.

Chapter 2 studies a recently described possible lead compound for a new $S1P_3$ antagonist. However, our study on this compound, BML-241, shows that besides the established inhibition of $S1P_3$-mediated changes in $[Ca^{2+}]_i$, this compound does not affect $S1P_3$ receptor-mediated decreases in cAMP accumulation. Futhermore, BML-241 inhibited changes in $[Ca^{2+}]_i$ mediated via other GPCRs, including the α_{1A}-adrenoceptor. The inhibitory effect on $[Ca^{2+}]_i$ is not mediated via a direct interaction with the receptor as BML-241 did not displace parozosin from the α_{1A}-adrenoceptor. From these results we concluded that BML-241 is a low potency inhibitor of $[Ca^{2+}]_i$ responses instead of a specific $S1P_3$ receptor antagonist.

In *chapter 3* we describe a new method to study S1P receptor internalization. The method is based on stable cell lines expressing a N-terminally HisG-tagged receptor, which can be visualized using various combinations of antibodies leading to diverse read outs. The best combination turned out to be a specific 1st antibody against the HisG-tag combined with a fluorescent 2nd antibody. The intensity of fluorescence is a direct measure for the level of receptor expression on the membrane. The developed method is quantitative and sensitive enough to measure differences in internalization induced by several ligands. It is also suitable to measure a large number of data points in a short time using a microplate reader.

Chapter 4 studies the vasoactive effects of S1P in a blood vessel. The effects of several S1P receptor ligands on the relaxation of a isolated rat aorta rings were combined with those on the signaling pathways of the two main cell types (smooth muscle

cells (VSMCs) and endothelial cells (ECs)). These effects were correlated to the specific receptor subtypes these ligands bind to and to their relative expression as established by qPCR. All tested compounds induced relaxation of rat aorta rings. In the VSMCs, mostly S1P induced elevation of $[Ca^{2+}]_i$, which in a vessel would result in contraction. In the ECs, most compounds inhibited cAMP accumulation, while hardly any effect was seen on $[Ca^{2+}]_i$ levels. From these date we concluded that in VSMCs, effects occur mostly through the $S1P_2$ receptor, while in the ECs, effects are a result of activation of the $S1P_1$ receptor. Therefore, since most compounds, with the exception of S1P itself, lack $S1P_2$ agonism, their net effect in the rat aorta will be relaxation.

The method described in chapter 3 has been utilized to study internalization of the $S1P_{1\text{-}3}$ receptors, which is described in *chapter 5*. There were striking differences between internalization of the $S1P_3$ receptor compared to the $S1P_1$ and $S1P_2$ receptor. Internalization of the $S1P_3$ receptor occurs within minutes, while the $S1P_1$ and $S1P_2$ receptor internalization requires approximately half an hour. Furthermore, the $S1P_3$ receptor internalizes at lower S1P-concentrations and shows a higher reduction of membrane receptors. Also, internalization for the $S1P_3$ receptor is influenced by G_i-inactivation, which does not influence internalization of the other two receptors.

In *chapter 6* the importance of the D/ERY motif in G-protein selectivity of S1P receptors is investigated. The influence of the 3rd amino acid in the D/ERY domain of the $S1P_{1\text{-}3}$ receptor was studied using site-directed mutagenesis with subsequent assessment of the effects of these mutations on the signaling via typical G_i and G_q-coupled pathways. It was found that for the $S1P_1$ receptor, mutations of this amino acid induced a ligand-dependent effect, in that for S1P and SEW2871, but not for FTY720-P, the G_i-mediated signaling was reduced. However, these mutations did not affect S1P-mediated changes in $[Ca^{2+}]_i$ in these cells. Mutating the ERH motif of the other two receptors did not induce major changes in signaling except for the $S1P_2$ receptor with an ERY motif instead of its native ERH motif. This mutated receptor showed an elevated S1P-induced increase of cAMP accumulation. From these data we concluded that the tyrosine residue of the D/ERY motif is not involved in restricting the $S1P_1$ receptor from activating more than one G-protein. However, since the mutation in this receptor differentially affected S1P and SEW2871 on one hand and FTY720-P mediated effects on the other hand we speculate that these

ligands probably induce different conformational states of the receptor which are differentially affected by the introduced mutations.

Several new S1P receptor ligands have recently become commercially available. In *chapter 7* we have studied their potency and efficacy in different signaling pathways via the $S1P_3$ receptor and in $S1P_3$ receptor internalization. In this regard, FTY720-P reacted as a full agonist when inhibiting cAMP accumulation, but was a partial agonist when inducing elevations in $[Ca^{2+}]_i$. Also, VPC23153 was more efficient in inhibiting cAMP accumulation than in elevating $[Ca^{2+}]_i$. Furthermore, a supposed antagonist for the $S1P_{1,3}$ receptor, VPC23019, behaved as a partial agonist in all three tested assays, being $[Ca^{2+}]_i$-signaling, cAMP-signaling and internalization. Another finding was that in high concentrations, S1P, in contrast to FTY720-P, also seemed to activate G_s-coupled pathways. We conclude from these data that several ligands show different response patterns via the $S1P_3$ receptor. This may be a clue towards ligand-directed signaling for the $S1P_3$ receptor, but that needs to be studied in more detail.

In *chapter 8*, besides the research in this thesis, other projects which did not result in a separate chapter are discussed. This includes our search for S1P receptor ligands in collaboration with the Free University and the Leiden University. The results were promising, but due to confidentiality reasons, this could not be further discussed. Another project involved a collaboration with the University of Würzburg, where they successfully developed a FlAsH-based FRET method to directly measure ligand/induced conformational changes in the adenosine A_{2A} receptor. We have made efforts to apply this method to the $S1P_1$ receptor. We obtained a FRET signal between the C-terminal CFG-tag and a built-in FlAsH-motif in the 3rd intracellular loop (IL3), but the FRET ratio did not change upon ligand stimulation. This was possibly due to a shorter, and thus less flexible, IL3 of the $S1P_1$ receptor compared to the adenosine A_{2A} receptor. However, when truncating the C-terminus of the $S1P_1$ receptor, we found that the part between aa 361 and 345 plays an important role in receptor surface expression. A final finding was made when the potency of ligands was compared in signaling transduction assays which have different incubation times. Pilot experiments have shown that in time, the S1P concentration declined, possibly due to degradation and/or uptake. Thus one should be careful not to underestimate the potency of a ligand when longer incubation times are used.

Samenvatting

Sphingosine-1-fosfaat (S1P) is een lipide dat in het lichaam diverse functies reguleert op cellulair niveau. Dit gebeurt via vijf G-eiwit gekoppelde receptoren (GPCRs), een soort zintuigen van de cellen, genaamd $S1P_{1-5}$. In dit proefschrift wordt eerst de ontwikkeling van enkele methodes en technieken besproken, welke nodig zijn om farmacologisch onderzoek te verrichten naar deze receptoren. Het tweede deel beschrijft het onderzoek naar de werking en regulatie van de receptoren zelf.

Hoofdstuk 1 is een introductie over GPCRs in het algemeen en S1P receptoren in detail. Het bespreekt de signalering, regulatie en effecten op een moleculair en cardiovasculair niveau. Tevens worden algemene methoden en technieken besproken die gebruikt worden bij het bestuderen van de signalering via S1P receptoren.

In *hoofdstuk 2* wordt een recent beschreven, nieuw ontwikkelde stof, genaamd BML-241, nader onderzocht. Deze stof werd geselecteerd op zijn eigenschappen om calcium signalering via de $S1P_3$ receptor, maar niet de $S1P_1$ receptor te remmen. Dit zou dus een mogelijk begin kunnen zijn van een nieuw te ontwikkelen $S1P_3$ antagonist (remmer). Echter, wij laten zien dat deze stof ook calcium signalering remt via andere GPCRs. Dit effect wordt niet veroorzaakt door een directe interactie met deze receptoren aangezien BML-241 geen competitie aanging met prazosine voor binding aan de α_{1A}-adrenoceptor. Daarnaast heeft BML-241 geen invloed op remming van de cAMP productie via de $S1P_3$ receptor. We concluderen dan ook dat het geen specifieke $S1P_3$ receptor remmer kan zijn, maar dat het meer lijkt op een algemene remmer van calcium signalering.

Hoofdstuk 3 beschrijft de ontwikkeling van een nieuwe methode om internalisatie van GPCRs te bestuderen en kwantificeren. Bij deze methode hebben een sequentie van 7 aminozuren (6xHisG-tag) die met een specifiek antilichaam herkend kunnen worden aan het N-terminale uiteinde van de receptor bevestigd. Er zijn diverse combinaties van antilichamen getest die met behulp van verschillende uitleesmethoden gekwantificeerd kunnen worden gebruikt. De combinatie van het eerste antilichaam met een tweede antilichaam met een fluorescerend label leverde de meest bruikbare methode op. Deze methode bleek zeer nuttig om een groot aantal monsters per keer te bestuderen en kwantificeren.

Hoofdstuk 4 beschrijft het onderzoek naar de effecten van S1P op het cardiovasculaire systeem. Er is gekeken naar de contractie (samentrekking) en relaxatie (ontspanning) van de aorta (de grote lichaamsslagader) van een rat onder invloed van diverse liganden van de $S1P_{1-3}$ receptoren, aangezien hiervan bekend is dat ze voorkomen in de aorta. Dit werd vervolgens gecombineerd met de effecten van deze stoffen op calcium en cAMP signalering in de twee meest voorkomende celtypen in de aorta, namelijk de endotheel cellen (ECs) (die de binnenkant van het bloedvat bekleden) en de gladde spiercellen (VSMCs). Dit werd tot slot gerelateerd aan de expressie van elk van de drie receptoren, gemeten via quantitatieve PCR. Er bleek dat in de VSMCs de effecten voornamelijk via de $S1P_2$ receptor worden veroorzaakt, wat resulteert in contractie. In de ECs worden de effecten veroorzaakt via de $S1P_1$ receptor, wat resulteert in relaxatie. Aangezien alle liganden met uitzondering van S1P zelf geen effecten via de $S1P_2$ receptor bewerkstelligen receptor, zal het totale effect op de aorta dus een relaxatie zijn.

De methode die we hebben ontwikkeld in hoofdstuk 3 is toegepast om de internalisatie van de $S1P_{1-3}$ receptoren te bestuderen, wat is beschreven in *hoofdstuk 5*. Er bleek een verschil te zijn in internalisatie van de $S1P_3$ receptor vergeleken met de $S1P_1$ en $S1P_2$ receptor. De $S1P_3$ receptor internaliseert al binnen enkele minuten na stimulatie, terwijl dit bij de andere twee receptoren ongeveer een half uur duurt. Bovendien bleek de internalisatie van de $S1P_3$ receptor ook nog eens te gebeuren bij een veel lagere S1P concentratie en er bleven veel minder $S1P_3$ receptoren in het celmembraan over dan $S1P_1$ of $S1P_2$ receptoren. Tevens bleek de internalisatie van de $S1P_3$ receptor te worden beïnvloed door het G_i-eiwit, wat bij de andere receptoren niet het geval was.

In *hoofdstuk 6* wordt het belang van een bepaald gebied (domein) van de S1P receptoren bestudeerd. Dit domein bestaat uit drie aminozuren, gecodeerd als D/ERY, en is bij diverse andere GPCRs al van belang gebleken voor signalering en constitutieve activiteit van de receptoren. Er is een opvallend verschil tussen de S1P receptoren op dit gebied. De $S1P_1$ receptor activeert namelijk alleen het G_i-eiwit en bevat een ERY domein, terwijl de andere receptoren meerdere G-eiwitten activeren en een ERH($S1P_{2,3}$), ERF ($S1P_4$) of ERS ($S1P_5$) domein bevatten. Zou dus deze tyrosine (Y) verantwoordelijk zijn voor het activeren van slechts het G_i-eiwit? Dit is onderzocht door het ERY motif van de $S1P_1$ receptor te vervangen

door het ERH motif van de $S1P_2$ en $S1P_3$ receptor en vervolgens te kijken naar het effect op de signalering, als gevolg van de activatie van de betreffende G-eiwitten. De signalering voor de $S1P_1$ receptor via het G_i-eiwit bleek te zijn verminderd, maar slechts voor 2 agonisten (activators) namelijk S1P en SEW2871 (die specifiek de $S1P_1$ receptor activeert) en niet voor een andere agonist FTY720-P. Echter, er werd geen verschil gevonden voor een signalerings route via het G_q-eiwit. Voor de andere receptoren bleken er geen grote verschillen te zijn tussen de oorspronkelijke en de gemuteerde receptoren. De $S1P_2$ receptor met een ERY domein in plaats van een ERH domein bleek cAMP concentraties in de cel flink te verhogen. Uit deze data concluderen we dat er geen verband is gevonden tussen het D/ERY domein in de S1P receptoren en hun specifieke G-eiwit signalering. Echter, er is een opvallend verschil in respons tussen de liganden S1P en SEW2871 aan de ene kant en FTY720-P aan de andere. Het zou kunnen dat FTY720-P een andere receptor conformatie induceert dan S1P en SEW2871 waardoor de mutaties in het D/ERY domein ook een andere effect op de signalering hebben.

Recentelijk zijn nieuwe liganden beschikbaar gekomen. In *hoofdstuk 7* zijn eigenschappen van deze stoffen onderzocht in diverse signaleringsroutes via de $S1P_3$ receptor. Dit resulteerde in de ontdekking dat een bepaald ligand, VPC23019, dat werd beschreven als een antagonist zich gedroeg als een partiële agonist in de geteste signalerings routes. Als extra werd nog gevonden dat S1P, maar niet FTY720-P, G_s-gekoppelde signalering bleek te activeren via de $S1P_3$ receptor. We concluderen dan ook dat de geteste liganden via de $S1P_3$ receptor verschillende signaleringspatronen induceren, wat mogelijk duidt op een nieuw fenomeen ook wel ligand-gestuurde signalering noemen.

Hoofdstuk 8 wordt naast het onderzoek dat beschreven staat in dit proefschrift, ook het onderzoek bediscussieerd dat nite heeft geleid tot een apart hoofdstuk. Dit omvat onze speurtocht naar nieuwe S1P receptor liganden in samenwerking met de Vrije Universiteit en de Universiteit Leiden. Wegens geheimhouding mogen deze veelbelovende resultaten echter niet in detail worden besproken.
Een ander project was een samenwerking met de Universiteit in Würzburg, waar een elegante FlAsH-based FRET methode was ontwikkeld om conformatie veranderingen geinduceerd door ligand binding in de adenosine A_{2A} receptor te meten. Wij hebben geprobeerd om deze methode ook toe te passen voor de

$S1P_1$ receptor. Ondanks dat we wel een FRET signaal konden meten tussen een C-terminale CFP en een FlAsH-motif in de 3e intracellulaire loop (IL3), bleek deze niet te veranderen onder invloed van ligand binding. Een mogelijke oorzaak hiervoor is de veel kortere en dus minder flexibele IL3 van de $S1P_1$ receptor in vergelijking met de adenosine A_{2A} receptor. Wel werd tijdens trunceren van de C-terminus van de $S1P_1$ receptor duidelijk, dat deze tussen aminozuur 361 en 345 een rol speelt bij expressie van de receptor aan de membraan.

Tot slot bemerkten we nog tijdens het vergelijken van liganden met behulp van verschillende signaaltransductie assays, dat de incubatie tijd erg van invloed kan zijn op de potentie van het ligand. Proef-experimenten hebben aangetoond dat de S1P concentratie daalt in de tijd, mogelijk door degradatie of opname door de cel. Men moet dan ook oppassen met het vergelijken van ligand eigenschappen met behulp van vershcillende assays, aangezien dit kan leiden tot een onderschatting van de werkelijke potency van het ligand.

List of publications

Jongsma M, Hendriks-Balk MC, Michel MC, Peters SL, Alewijnse AE. BML-241 fails to display selective antagonism at the sphingosine-1-phosphate receptor, $S1P_3$. *Br J Pharmacol.* 2006; **149**:277-282

Michel MC, Mulders ACM, **Jongsma M**, Alewijnse AE, Peters SLM. Vascular effects of sphingolipids. *Acta Paediatr Suppl.* 2007;**96**:44-48

Jongsma M, Florczyk UM, Hendriks-Balk MC, Michel MC, Peters SLM, Alewijnse AE. Optimising a rapid, non-radioactive method to quantify internalisation of G-protein-coupled receptors. *Manuscript submitted*

Jongsma M, Hendriks-Balk MC, Mathy M-J, Michel MC, Peters SL, Alewijnse AE. BML-241, a specific $S1P_3$ receptor antagonist? *Naunyn-Schmiedeberg's Archives of Pharmacology.* 2007; **375**:329-336

Jongsma M, Mathy MJ, Van Loenen PB, Hajji N, Michel MS, Peters SLM, Alewijnse AE. Pharmacological and biochemical characterization of the vasoactive properties of sphingosine-1-phosphate receptors in rat aorta. *Manuscript submitted*

Jongsma M, Michel MC, Peters SLM, Alewijnse AE. Differential agonist-induced internalization of sphingosine-1-phosphate receptor subtypes 1, 2 and 3. *Manuscript in preparation*

Jongsma M, Den Boon FS, Van Loenen PB, Michel MC, Peters SLM , Alewijnse AE. Involvement of the third amino acid of the D/ERY motif in G-protein selectivity of S1P receptors. *Manuscript in preparation*

Jongsma M, Van Unen J, Hajji N, Michel MC, Peters SLM, Alewijnse AE. Differential response patterns of several ligands at the sphingosine-1-phosphate receptor subtype 3. *Manuscript in preparation*

Dankwoord

Tijdens een promotietraject van vier jaar kom je in aanraking met vele mensen die allen op hun eigen wijze hun bijdrage leveren. Het resulteert uiteindelijk in dit proefschrift waar slechts mijn naam op de omslag te lezen valt, maar dit betekent natuurlijk niet dat dit een eenmans project is geweest. Zonder de hulp van velen, maar enkelen in het bijzonder had het onderzoek nooit geleid tot dit resultaat.
Een volledig nieuwe afdeling, waarin alles nog moest worden geregeld, besteld, getest en opgezet, was de omgeving waarin ruim vier jaar geleden zes nieuwe AIO's zijn gestart, waarvan ik er een was. Uiteraard wordt hierbij veel gevallen en weer opgestaan. In mijn opinie is het onjuist om pas achteraf te laten blijken dat mensen belangrijk voor je zijn op bepaalde momenten in je leven. Het is juist zeer wenselijk om ze ook op de betreffende momenten te laten weten dat je de ontvangen hulp en steun waardeert. Ik hoop dat ik dat dan ook gedaan heb en dat de mensen die hieronder hun naam zullen lezen dat kunnen beamen. Ik houd niet van volgordes, rangen en standen, maar voor mij is iedereen gelijk. Echter, in een dankwoord ontkom je niet aan een volgorde, maar voor mij is die dus willekeurig.

Laat ik dan ook beginnen met mijn promotor, prof. Martin Michel te bedanken. Martin, een nieuwe afdeling is er een die ruimte nodig heeft om uit te kristalliseren tot een werkomgeving waarin een ieder zich kan ontwikkelen tot dat waar hij het beste in is en de grootste bijdrage kan leveren. Deze ruimte heb je zeker gegeven. Daarnaast zal jouw ongeëvenaarde omgang met teksten, het eenvoudig omschrijven van iets waar je zelf niet meer uitkomt, mij bijblijven. Zeker het laatste half jaar is dit van onmisbaar belang geweest bij het schrijven van in totaal maar liefst zes manuscripten, iets waar we best trots op mogen zijn denk ik.

Vervolgens kom ik terecht bij de persoon die me wat betreft dagelijks werk het meest heeft begeleid, Astrid Alewijnse. Astrid, ook voor jou die nieuwe afdeling met al die onervaren mensen. Het waren geen makkelijke jaren. Grote frustraties werden afgewisseld met mooie ontdekkingen. Jouw nooit aflatende energie, inzet en ambitie zijn een inspiratie voor me geweest. Zonder jou was dit proefschrift er nooit gekomen. Je hebt me kansen gegeven om me te ontwikkelen, in binnen en buitenland en ook al is nog niet alles vlekkeloos, het is zeker stukken beter dan vier jaar geleden. Je zei ooit dat het jouw taak was om je AIO's op een goede plek te

laten terechtkomen. In mijn geval is je dat goed gelukt. Mijn postdoc plek op het NKI/AVL is in mijn ogen een directe verdienste van jou. Ik hoop dat je na jaren van investeren nu kunt gaan oogsten met mooie publicaties met de nieuwe lichting AIO's. Heel veel succes hierbij en ik hoop dat we elkaar in de toekomst nog kunnen helpen.

De volgende in het ´rijtje´ is mijn tweede copromotor, Stephan Peters. Stephan, hoewel je bij mijn onderzoek naar buiten toe misschien wat meer op de achtergrond stond waren de discussies toch altijd zeer nuttig. Jouw uitgebreide kennis van de farmacologie verbaasde me telkens weer, hoewel natuurlijk niet elk voorstel daadwerkelijk werd getest (cafeïne bijvoorbeeld!). Jouw input was niet alleen puur wetenschappelijk, zo zorgde je ook voor de ontspanning op de afdeling. Samen met Astrid heb je ook bewezen dat een relatie met een collega prima mogelijk is en zelfs voor een erg goede balans kan zorgen. Een tip nog, pas bij het squashen op voor het racket van de tegenstander, zeker als deze van huis uit een tennisser is…

Mariëlle, collega AIO vanaf bijna het eerste uur, paranimf en helft van de 'kweekfabriek'. We hebben wel eens gezegd dat we zonder elkaar beiden waren gestopt. In het begin was het zwaar, maar tijdens de lange kweeksessies, groeiden we toch naar elkaar toe, tegen ieders verwachting in. En juist dat maakte dat we doorgingen, elkaar steunend. Ik waardeer je eerlijkheid en inzet en het is fijn om een collega te hebben die werkt op een zelfde manier als ikzelf. Ik hoop dat jouw project nog de resultaten gaat opleveren die je trots maken op de hoeveelheid werk die je hebt verzet.

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En dan zijn er nog altijd de mensen buiten het werk die op een ander vlak voor mij belangrijk zijn.

Lukas, jouw kijk op alledaagse dingen is voor mij vaak verhelderend. Sinds ik je leerde kennen tijdens mijn stage op het RIVM heb je me op vele vlakken van wijze raad voorzien, maar hebben we nog veel meer ontspannende uren doorgebracht. Tijdens de ontelbare spellen die we inmiddels hebben gespeeld gaan we amper een gelegenheid uit de weg om elkaar te belazeren, maar daarbuiten is het volledig omgekeerd. Ik ben blij je als vriend te hebben.

Christie, even though you are so far away, since we met in the lab in Stellenbosch, we have become close friends. I am glad you came to visit me last year so I could show you my country and I am looking forward to meeting you again in February. I thank you for always listening (or reading) to my stories and giving me your advise, never without your remarkable sense of humor. I am glad we stayed in touch and hope to continue this for much, much longer

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Maikel

Curriculum Vitae

The author of this thesis was born on June 6th, 1977 in Diepenheim. At the age of 10, he moved to Hengelo where he graduated from his pre-university education at the R.K. Lyceum de Grundel at the age of 17. He started his study of Chemical Engineering at the University of Twente in 1994, from which he did not graduate. The following year, he started his Medical Laboratory Education at the University of Professional Education in Enschede. This was successfully concluded in 1999 including a 10 month internship, working on *Haemophilus influenzae* at the National Institue for Public Health and the Environment in Bilthoven, under supervision of Dr. P. van Ulsen. In that same year he started his Biology education at the University of Utrecht, which included a 9 month practical period at the University of Stellenbosch (South Africa) under supervision of Prof.dr. J.L. Snoep. There he worked on glucose-dependent growth of *Saccharomyces cerevisiae*. After graduation in December 2002, he started in the summer of 2003 to work in the lab of Prof.dr. M.C. Michel, under supervision of Dr. A.E. Alewijnse at the department of Pharmacology and Pharmacotherapy at the Academic Medical Center in Amsterdam. It was here that he performed the work described in this thesis. At the Keystone meeting in Taos (NM, USA) on Bioactive Lipids in the Lipidomics Era in 2007 he was awarded a Keystone Symposia Scholarship for his work on internalization of sphingosine-1-phosphate receptors. In August 2007 he started as a postdoc in the lab of Prof.dr. W.H. Moolenaar at the Netherlands Cancer Institute to work on lysophophatidic acid receptors.