Regulation of cardiovascular GPCR signalling
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Differential agonist-induced regulation of the sphingosine-1-phosphate receptor subtypes 1 and 3 (S1P₁ and S1P₃)

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ABSTRACT

Regulation of sphingosine-1-phosphate (S1P) receptors is believed to be the mechanism of action of the immunosuppressive prodrug FTY720. Its active metabolite FTY720-P can induce internalization of S1P_1 receptors in HEK293 cells which is followed by irreversible receptor degradation, thus displaying “functional antagonistic” properties on S1P_1 receptors. In contrast, the natural ligand S1P causes reversible S1P_1 receptor internalization. It is unknown whether this differential regulation by S1P and FTY720-P also occurs in other cell lines and with other S1P receptor subtypes. Therefore, we investigated the S1P and FTY720-P-induced regulation and subsequent recycling of the S1P_3 receptor, a receptor involved in the cardiac side effects of FTY720, in CHO-Flp-In cells. We show that, similar to previous findings, S1P_1 receptors expressed in CHO-Flp-In cells are also differentially regulated by S1P and FTY720-P. However, the time- and concentration-dependent internalization of S1P_3 receptors upon stimulation with either, S1P or FTY720-P, was reversible indicating that the S1P_3 receptor is not differentially regulated by these ligands. We conclude that the S1P_1-mediated immunosuppressive effects of FTY720 might involve sustained internalization and down-regulation of the receptor, whereas this mechanism apparently does not underlie the S1P_3-mediated cardiac effects of FTY720.
INTRODUCTION

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid that mediates many important cellular responses such as proliferation, migration, barrier function and lymphocyte trafficking (Brinkmann, 2007; Brinkmann and Baumruker, 2006; Peters and Alewijnse, 2007). S1P can be generated from membrane lipids by the concerted action of several enzymes involved in sphingolipid metabolism (Hannun and Obeid, 2008; Tani et al., 2007). Most of the effects of S1P are mediated by five distinct G protein-coupled receptors (GPCRs) of the S1P receptor family, S1P₁-S1P₅ (previously endothelial differentiation gene (EDG) receptor-1, 5, 3, 6, 8, respectively) (Chun et al., 2002). The S1P₁, S1P₂ and S1P₃ receptors are ubiquitously expressed and are the predominant S1P receptors in the cardiovascular system (Alewijnse et al., 2004; Brinkmann and Baumruker, 2006). S1P₄ and S1P₅ receptor expression is more limited: the S1P₄ receptor is mainly expressed in the lung and lymphoid tissue (Graler et al., 1998; Jolly et al., 2002) and the S1P₅ receptor is predominantly expressed in the brain (Im et al., 2000; Terai et al., 2003).

Activation of a GPCR will, via the activation of a G protein, result in the modulation of specific signalling pathways. Furthermore, GPCR activation will initiate several regulatory processes, some of which affect receptor responsiveness directly (Bockaert et al., 2004; Ferguson, 2001; Hendriks-Balk et al., 2008; Marchese et al., 2008). One of these processes involves GPCR phosphorylation by GPCR kinases and the subsequent binding of arrestin to the receptor, which leads to uncoupling of the receptor from the G proteins thereby terminating the signalling (Shenoy and Lefkowitz, 2003). Another regulatory process is receptor internalization, referring to the sequestration of the receptor in small membrane vesicles (Hanyaloglu and von Zastrow, 2008). Internalization can be followed either by the irreversible degradation of the receptor and thus receptor down-regulation or by recycling of the receptor to the cell surface (Drake et al., 2006; Ferguson, 2001; Gaborik and Hunyady, 2004; Marchese et al., 2008).

For the S1P receptor family, detailed knowledge on the regulation of most of the receptor subtypes is still missing except for the S1P₁ receptor subtype. Interestingly, internalization and degradation of this receptor subtype is suggested to be the mechanism of action of FTY720 (fingolimod), an immunosuppressant, which is in clinical trials for the treatment of multiple sclerosis (Baumruker et al., 2007; Dev et al., 2008; Hiestand et al., 2008). FTY720 is a prodrug, which can be phosphorylated by sphingosine kinase 2 to FTY720-P, which is a potent agonist for all S1P receptors except the S1P₂ receptor (Brinkmann et al., 2002). The FTY720-P-induced lymphopenia as a result of lymphocyte sequestration in thymus and peripheral lymphoid organs (Mandala et al., 2002; Matloubian et al., 2004) is predominantly mediated via the S1P₁ receptor on lymphocytes. Although FTY720-P acts, like S1P, as S1P₁ agonist, its immunosuppressant effect is suggested to be caused by its “functional antagonistic” properties. Upon S1P₁ receptor activation both S1P and FTY720-P induce internalization of this receptor (Graler and Goetzl, 2004; Jo et al., 2005; Oo et al., 2007). However, the S1P-induced internalization of
the S1P₁ receptor is followed by a recycling of the receptor to the cell surface (Jo et al., 2005; Oo et al., 2007) whereas FTY720-P induces irreversible degradation of the internalized S1P₁ receptor in HEK293 cells resulting in receptor down-regulation (Graler and Goetzl, 2004; Oo et al., 2007).

Because FTY720 is, as mentioned before, not a specific S1P₁ receptor agonist this compound may, besides its beneficial S1P₁ receptor-mediated immunosuppressant effect in multiple sclerosis, cause acute and/or chronic side-effects via the activation and/or regulation of other S1P receptor subtypes. For example, activation and/or regulation of the S1P₃ receptor may have serious cardiovascular side-effects as increasing evidence suggests a protective role for this receptor subtype in the cardiovascular system (reviewed by (Alewijnse and Peters, 2008)). Indeed, FTY720 has been shown to induce a profound bradycardia (Budde et al., 2002), which is suggested to be S1P₃ receptor-mediated (Forrest et al., 2004; Sanna et al., 2004). At the moment it is unknown whether FTY720-P also induces irreversible degradation of the S1P₃ receptor subtype. In this study we first investigated whether the differential regulation of the S1P₁ receptor by S1P and FTY720-P also occurs in CHO-Flp-In cells. Furthermore, we investigated the regulation and subsequent recycling or degradation of the S1P₃ receptor in CHO-Flp-In cells to answer the question whether this receptor is, like the S1P₁ receptor, also differentially regulated by S1P and FTY720-P.

**MATERIALS AND METHODS**

**Materials**

CHO-Flp-In cells, pcDNA3.1HisA/B/C, pcDNA5/FRT/TO, pOG44, Lipofectamine™ 2000, OptiMEM, F-12 nutrient mixture (Ham), penicillin/streptomycin, fetal bovine serum (FBS), trypsin/EDTA, phosphate buffered saline (PBS), Alexa Fluor 488® goat anti-mouse IgG, anti-HisG antibody (mouse monoclonal IgG₂a) and ProLong® Gold antifade reagent were obtained from Invitrogen (Breda, The Netherlands). Hygromycin B was from PAA (Coelbe, Germany). Activated charcoal, Tween-20 and fatty acid-free bovine serum albumin (BSA) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Formaldehyde solution (16%, methanol-free) was obtained from Pierce Biotechnology (via Perbio Science, Etten-Leur, The Netherlands). S1P was obtained from Avanti Polar Lipids Inc (via Instruchemie, Delfzijl, The Netherlands) and dissolved at a concentration of 100 µM in 4 mg/ml BSA in sterile water. FTY720-P (2-Amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol mono(dihydrogen phosphate) ester) was synthesized according to previously described methods (Albert et al., 2005) and dissolved at a concentration of 10 mM in DMSO with a droplet HCl (0.1 M). The expression vectors pcDNA3.1-S1P₁ and pcDNA3.1-S1P₃ were purchased from UMR cDNA Resource Center (Rolla, MO, USA).
Cell culture

CHO-Flp-In cells stably expressing the His-tagged human S1P₁ (Jongsma et al., 2007a) or S1P₃ receptor were used. The cDNA of the S1P₃ receptor obtained from UMR contained a point mutation G⁹⁶₂A (which results in amino acid change R³²¹Q), which was removed by PCR using the following primers (forward: TG CCTGGTCAGGGGCGGGGGCGCC, reverse: CGGGCCCCGGCCCCCTGACCAGGCA). The S1P₃ receptor-coding DNA in the resulting plasmid as well as the stable cell line constructed with this plasmid have been confirmed by sequencing. CHO-Flp-In cells stably expressing His-tagged S1P₃ receptors were constructed in a similar way as described before (Jongsma et al., 2006). The His-tag has no influence on signal transduction induced via this receptor (data not shown).

CHO-Flp-In cells stably expressing the His-tagged S1P₁ or S1P₃ receptor were passaged 1:3 every 2 or 3 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 FBS. All cell lines were cultured at 37 °C in humidified air containing 5% CO₂.

Immunocytochemistry

CHO-Flp-In cells were seeded in black 96 well plates at 40,000 cells/well (receptor internalization) or in 8 well chamber slides at 40,000 cells/well (receptor re-appearance) two days before the measurement. After serum starvation overnight, cells were stimulated with S1P or FTY720-P at the indicated concentrations at 37°C for the indicated time. To measure receptor re-appearance, cells were washed three times with serum-free medium and incubated at 37°C in serum-free medium for the indicated time.

Directly after stimulation (receptor internalization) or after incubation in medium (receptor re-appearance), the cells were fixated with cold 4% formaldehyde in PBS for 10 min on ice. After washing three times with PBS, the cells were blocked by 4% BSA in PBS for 10 min at room temperature and subsequently incubated with anti-HisG (1:100) in PBS for 1 h at room temperature while shaking. Thereafter, the cells were washed three times with PBS and incubated with Alexa Fluor 488® goat anti-mouse (1:500) in PBS for 1 h at room temperature while shaking in the dark. After washing, fluorescence (excitation 490 nm, emission 535nm) in the 96 well plates was measured using a plate reader (Victor 2, Wallac, Perkin Elmer). Chamb erslides were mounted with ProLong® Gold antifade reagent and fluorescence was measured using fluorescence microscopy (Nikon Eclipse TE2000-U).

Data analysis

For internalization measurements, data were normalized to the fluorescence observed in the unstimulated situation, which was set at 100%. The t½ is visually determined as the time to
reach 50% of the maximal internalization. Data are expressed as mean ± S.E.M. of at least three independent experiments. A Student’s t-test was applied where appropriate. A P<0.05 was considered as statistically significant.

**Fig. 1.** Cell surface expression of the His-tagged S1P\(_1\) receptor in CHO-Flp-In cells. Cells were treated with S1P (1 µM), FTY720-P (10 µM) or their respective vehicles BSA and DMSO, for 30 min and washed. Some cells were stained directly whereas others are incubated with serum-free medium for 4 h and stained as described. Images shown are representative for at least three independent experiments.
Chapter 3

RESULTS AND DISCUSSION

The immune modulator FTY720 is the first ligand targeting S1P receptors in clinical trials for the treatment of multiple sclerosis (Baumruker et al., 2007; Dev et al., 2008; Hiestand et al., 2008). The immunosuppressant effect of FTY720 is attributed to the FTY720-induced lymphopenia, which is predominantly mediated via S1P₁ receptors on lymphocytes (Mandala et al., 2002; Matloubian et al., 2004).

We previously showed that the S1P₁ receptor is internalized upon agonist stimulation using a quantitative and sensitive measurement we have developed in our laboratory (Jongsma et al., 2007a). Several other studies also report internalization of the S1P₁ receptor induced by S1P and FTY720 (Graler and Goetzl, 2004; Jo et al., 2005).

Following internalization GPCRs can either be recycled to the cell surface or degraded irreversibly (Drake et al., 2006; Gaborik and Hunyady, 2004). Interestingly, it has been shown in HEK293 cells expressing the S1P₁ receptor that upon stimulation this receptor is differentially regulated by S1P and FTY720-P (Oo et al., 2007). Internalized S1P₁ receptors were shown to be recycled to the membrane within 2 h after S1P stimulation, whereas after FTY720-P stimulation no recycling of receptors could be observed up to 8 h but instead the receptors

Fig. 1. Vervolg.
were shown to be degraded (Oo et al., 2007). Our study in CHO-Flp-In cells stably expressing the human S1P\textsubscript{1} receptor confirms the findings by Oo et al. in HEK293 cells indicating that the differential effects of S1P and FTY720-P on regulation are not cell type dependent. We show that both S1P and FTY720-P induced internalization of the S1P\textsubscript{1} receptor after 30 min of stimulation (Fig. 1). Within 4 h of recovery the S1P-internalized S1P\textsubscript{1} receptors re-appeared at the plasma membrane (Fig. 1). However, after FTY720-P stimulation the S1P\textsubscript{1} receptor was still internalized after 4 h of recovery (Fig. 1). Even after 6 h of recovery the receptors did not re-appear at the membrane (data not shown). Unfortunately, we were not able to directly quantify the experiments on receptor recycling using our earlier described method (Jongsma et al., 2007a) due to some technical problems. More specifically, since the washing procedures used after ligand stimulation resulted in a loss of cells, the membrane expression could not be reliably quantified with a microplate reader, as the total fluorescence observed per well is also dependent on the amount of cells per well. We, therefore, choose to study the recycling or re-appearance at the cell surface using fluorescence microscopy, which makes it possible to look at specific areas in the well. As our findings using this method were similar to previous findings on the S1P\textsubscript{1} receptor (Oo et al., 2007), we conclude that our method can be used to determine whether human S1P\textsubscript{3} receptors are differentially regulated by S1P or FTY720-P.

Internalization of the S1P\textsubscript{3} receptor upon stimulation with S1P has been demonstrated previously (Jongsma et al., 2007b; Rutherford et al., 2005). FTY720 was reported to have no effect on the regulation of the S1P\textsubscript{3} receptor (Graler and Goetzl, 2004) but as the unphosphorylated form of FTY720 only has moderate affinity for the S1P\textsubscript{3} receptor (Dev et al., 2008) this is not surprising. Very recently, just after completion of our study, it has been shown in HTC4 cells that also FTY720-P, at a concentration of 300 nM, did not induce internalization of the S1P\textsubscript{3} receptor after 30 min of stimulation (Sensken et al., 2008). However, in the present study we clearly demonstrate that both S1P and FTY720-P are able to induce internalization of the S1P\textsubscript{3} receptor (Fig. 2 and 3). The decrease in cell surface expression of the S1P\textsubscript{3} receptor, measured as a decrease in membrane fluorescence, was very rapid after stimulation with 1 \(\mu\)M S1P (\(t_{1/2} = 1.1 \pm 0.2\) min, n=4) and was maximal after about 10 min of stimulation (Fig. 2A). Stimulation with FTY720-P (10 \(\mu\)M) induced internalization at a significant slower rate (\(t_{1/2} = 8.5 \pm 0.2\) min, n=3, P<0.05) and the internalization was maximal after 20 min of stimulation (Fig. 2A). Importantly, parallel stimulations with vehicle did not result in a decrease in membrane fluorescence (Fig. 2A). In addition, the internalization induced by S1P and FTY720-P was shown to be concentration-dependent (Fig. 2B). A 30 min stimulation with increasing concentrations of S1P or FTY720-P decreased the cell surface expression of the S1P\textsubscript{3} receptor with similar efficacy and a slight difference in potency (\(pEC_{50} = 8.1 \pm 0.3\) and 7.8 \(\pm\) 0.2, respectively, n=3) (Fig. 2B).

The discrepancy between our results on FTY720-P-induced internalization and those of Sensken et al. (Sensken et al., 2008) may be explained by the differences in the FTY720-P concentration used (300 nM versus 10 \(\mu\)M). At lower concentrations the kinetics of internalization
can be expected to be slower and, therefore, the amount of receptor internalization at 30 min is probably still too low to be measured when using 300 nM FTY720-P. In addition, although receptor internalization is known to be temperature dependent process Sensken et al. (Sensken et al., 2008) did not mention at which temperature the studies on S1P$_3$ internalization were performed. The fact that both S1P and FTY720-P induced S1P$_3$ receptor internalization in our cell system enabled us to investigate whether the S1P$_3$ receptor is differentially regulated by S1P and FTY720-P after internalization. Because of the small difference in the potency to induce S1P$_3$ receptor internalization between the two compounds we choose to use a 10-fold higher concentration of FTY720-P than of S1P for the re-cycling experiments.

As shown in Fig. 3, both S1P and FTY720-P decreased the cell surface expression of the S1P$_3$ receptor after 30 min of stimulation, which confirms the quantitative data shown in Fig. 2. Interestingly, as observed for the S1P$_1$ receptor (Fig. 1), the S1P$_3$ receptor re-appeared at the cell surface within 4 h after stimulation with S1P (Fig. 3). In contrast to the findings on the S1P$_1$ receptor, S1P$_3$ receptors stimulated with FTY720-P also re-appeared at the cell surface within 4 h after stimulation (Fig. 3). Thus, FTY720-P does not induce the degradation of the S1P$_3$ receptor, but rather shows the same effects as S1P on S1P$_3$ receptor regulation after internalization.

In summary, the present study shows that, similar to findings in HEK293 cells, S1P$_1$ receptors expressed in CHO-Flp-In cells are differentially regulated after stimulation by S1P or FTY720-P. Furthermore, this study demonstrates that this differential regulation by S1P and FTY720-P cannot be extended to the S1P$_3$ receptor. In contrast to the S1P$_1$ receptor, S1P$_3$ receptors re-appeared at the cell surface both after S1P and FTY720-P stimulation at similar recovery times. Therefore, whereas sustained internalization and down-regulation of the S1P$_1$ receptor might underlie the immunosuppressive effect of FTY720, this mechanism is not involved in the S1P$_3$-mediated effects of FTY720.

![Fig. 2. Internalization of the His-tagged S1P$_1$ receptor in CHO-Flp-In cells. A) Time course of the effect of S1P and FTY720-P stimulation, or their respective vehicles BSA and DMSO, on cell surface expression of the S1P$_1$ receptor. Cells were stimulated with S1P (1 μM) or FTY720-P (10 μM) for the indicated times. B) Concentration response curves for the S1P$_1$ receptor internalization induced by 30 min stimulation with S1P and FTY720-P. Data represent means ± S.E.M. of at least three independent experiments.](image)
**Fig. 3.** Cell surface expression of the His-tagged S1P₁ receptor in CHO-Flp-In cells. Cells were treated with S1P (1 μM), FTY720-P (10 μM) or their respective vehicles BSA and DMSO, for 30 min and washed. Some cells were stained directly whereas others are incubated with serum-free medium for 4 h and stained as described. Images shown are representative for at least three independent experiments.
Differential agonist-induced regulation of the S1P1 and S1P3 receptor subtypes

Fig. 3. Vervolg.
REFERENCES


Differential agonist-induced regulation of the S1P₁ and S1P₃ receptor subtypes