Biochemical Pharmacology 81 (2011) 617–625

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Cis-4-methylsphingosine is a sphingosine-1-phosphate receptor modulator

Michael ter Braak [a], Ralf Frederik Claas [b], Bianca Hegen [a], Sandra Labocha [c], Nerea Ferreirós [c], Josef Pfeilschifter [b], Andrea Huwiler [d], Gerhild van Echten-Deckert [e], Dagmar Meyer zu Heringdorf [b,*]

[a] Institut für Pharmakologie, Universitätsklinikum Essen, Germany
[b] Institut für Allgemeine Pharmakologie und Toxikologie, Klinikum der Goethe-Universität Frankfurt am Main, Germany
[c] Institut für Klinische Pharmakologie, Klinikum der Goethe-Universität Frankfurt am Main, Germany
[d] Institute of Pharmacology, University of Bern, Friedbühlstrasse 49, CH-3010 Bern, Switzerland
[e] Kekulé-Institut, LIMES Membrane Biology and Lipid Biochemistry, Universität Bonn, Germany

ARTICLE INFO

Article history:
Received 27 September 2010
Accepted 2 December 2010
Available online 14 December 2010

Keywords:
Sphingosine-1-phosphate
Lysophospholipid receptors
GPCR
Sphingosine kinase
FTY720

ABSTRACT

Sphingosine-1-phosphate (S1P) acts as high affinity agonist at specific G-protein-coupled receptors, $S1P_{1-5}$, that play important roles e.g. in the cardiovascular and immune systems. A S1P receptor modulating drug, FTY720 (fingolimod), has been effective in phase III clinical trials for multiple sclerosis. FTY720 is a sphingosine analogue and prodrug of FTY720-phosphate, which activates all S1P receptors except $S1P_2$ and disrupts lymphocyte trafficking by internalizing the $S1P_1$ receptor. Cis-4-methylsphingosine (cis-4M-Sph) is another synthetic sphingosine analogue that is readily taken up by cells and phosphorylated to cis-4-methylsphingosine-1-phosphate (cis-4M-S1P). Therefore, we analysed whether cis-4M-Sph interacted with S1P receptors through its metabolite cis-4M-S1P in a manner similar to FTY720. Indeed, cis-4M-Sph caused an internalization of S1P receptors, but differed from FTY720 as it acted on $S1P_2$ and $S1P_3$ and only weakly on $S1P_1$, while FTY720 internalized $S1P_1$ and $S1P_3$ but not $S1P_2$. Consequently, pre-incubation with cis-4M-Sph specifically desensitized S1P-induced $[Ca^{2+}]_i$ increases, which are mediated by $S1P_2$ and $S1P_3$, in a time- and concentration-dependent manner. This effect was not shared by sphingosine or FTY720, indicating that metabolic stability and targeting of $S1P_2$ receptors were important. The desensitization of S1P-induced $[Ca^{2+}]_i$ increases was dependent on the expression of SphKs, predominantly of SphK2, and thus mediated by cis-4M-S1P. In agreement, cis-4M-S1P was detected in the supernatants of cells exposed to cis-4M-Sph. It is concluded that cis-4M-Sph, through its metabolite cis-4M-S1P, acts as a S1P receptor modulator and causes S1P receptor internalization and desensitization. The data furthermore help to define requirements for sphingosine kinase substrates as S1P receptor modulating prodrugs.

1. Introduction

The sphingosine analogue, FTY720 (fingolimod), has recently caused interest as a potent and efficient modulator of the immune system [1]. Its efficacy in relapsing–remitting multiple sclerosis has been demonstrated in phase III clinical trials with more than 1500 treated patients [2,3]. Compared to other immunosuppressive drugs, FTY720 has a unique mechanism of action as its phosphate interacts with G-protein-coupled sphingosine-1-phosphate (S1P) receptors [4,5]. S1P is a ubiquitous lipid mediator that on the cellular level regulates growth and survival, migration, adhesion and Ca^{2+} homeostasis. S1P is generated from sphingosine by sphingosine kinases (SphKs), of which two isoforms have been identified (for review, see [6]). Degradation of S1P occurs via dephosphorylation by specific S1P phosphatases or lipid phosphate phosphatases, or via irreversible cleavage catalysed by S1P lyase (reviewed in [7,8]). G-protein coupled S1P receptors are nearly ubiquitously expressed and regulate for example lymphocyte trafficking, vasculogenesis, vascular contractility and permeability, heart beat, neural tube formation or inner ear function [9,10]. There are five S1P receptor subtypes, $S1P_{1-5}$, that can have overlapping or opposing functions [7]. S1P furthermore has

Abbreviations: BSA, bovine serum albumin; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; Cis-4M-S1P, cis-4-methylsphingosine-1-phosphate; Cis-4M-Sph, cis-4-methylsphingosine; LC–MS/MS, liquid chromatography–tandem mass spectrometry; LPA, lysophosphatidic acid; MEFs, mouse embryonic fibroblasts; PTX, pertussis toxin; S1P, sphingosine-1-phosphate.

* Corresponding author at: Institut für Allgemeine Pharmakologie und Toxikologie/ZAFES, Klinikum der Goethe-Universität, Theodor-Stern-Kai 7, D-60590 Frankfurt am Main, Germany. Tel.: +49 69 6301 3906; fax: +49 69 6301 7942.
E-mail addresses: Michael.Terbraak@uk-essen.de (M. ter Braak), Claas@med.uni-frankfurt.de (R.F. Claas), Bianca.Hegen@uk-essen.de (B. Hegen), labocha@med.uni-frankfurt.de (S. Labocha), FerreirosBouzas@em.uni-frankfurt.de (N. Ferreirós), Pfeilschifter@em.uni-frankfurt.de (J. Pfeilschifter), huwiler@pki.unibe.ch (A. Huwiler), g.echten.deckert@uni-bonn.de (G. van Echten-Deckert), heringdorf@med.uni-frankfurt.de (D. Meyer zu Heringdorf).

0006-2952/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2010.12.002

intracellular target sites as it directly inhibits histone deacetylases [11] or mobilizes stored Ca^{2+} independently of G-protein-coupled S1P receptors [12].

FTY720 is used by the S1P signalling toolkit as a surrogate for sphingosine. It is phosphorylated by SphK2, and FTY720-phosphate acts as an agonist at all S1P receptors except $S1P_2$ [4,5]. The immunosuppressive effect of FTY720 is caused by internalization of the $S1P_1$ receptor, which renders lymphocytes insensitive to the gradient of S1P between blood and lymphoid tissue and thereby inhibits the emigration of lymphocytes from lymphatic tissues [13–16]. Side effects of FTY720 such as bradycardia, increase in mean forced expiratory volume in 1 s and mild increase in blood pressure, are most likely caused by agonistic activity of FTY720-phosphate at $S1P_3$ receptors. The pharmaceutical development aims at specific $S1P_1$ receptor agonists for mimicking the immunosuppressive activity of FTY720 with less side effects caused by interaction with S1P receptors other than $S1P_1$. Another approach tries to identify other SphK substrates as S1P receptor prodrugs [17]. Cis-4-methylsphingosine (cis-4M-Sph) is such a sphingosine analogue that is readily taken up by cells and phosphorylated to cis-4-methylsphingosine-1-phosphate (cis-4M-S1P) [18,19]. A systematic analysis of sphingosine analogues revealed that after incubation with cis-4M-Sph, but not trans-4-methylsphingosine, cis-5-methylsphingosine or trans-5-methylsphingosine, the phosphate accumulated within mouse cerebellar neurons [18]. Since all of the mentioned sphingosine analogues were phosphorylated by SphKs, the analogues with "trans" configuration even better, it was concluded that cis-4M-S1P accumulated because it poorly qualified as substrate for S1P lyase [18]. Cis-4M-S1P also accumulated in neuroblastoma cells and Swiss 3T3 fibroblasts treated with cis-4M-Sph, but to a lesser extent [19,20]. Biological activity of cis-4M-Sph was observed in these earlier studies at the relatively high concentration of 10 μM, at which this compound inhibited serine palmitoyltransferase by about 50% in primary cultured neurons [18] but not in other cell types (van Echten-Deckert, unpublished data). Incubation with 10 μM cis-4M-Sph furthermore induced apoptosis in neuroblastoma cells and in primary cultured neurons, but mimicked the mitogenic effect of S1P in Swiss 3T3 fibroblasts [19]. Recently, it was shown that the neurotoxic effect of 10 μM cis-4M-Sph was dependent on SphK2 and thus mediated by the accumulating phosphate [21]. Thus, cis-4M-Sph displays some similarities with FTY720 as it is phosphorylated intracellularly and the phosphate accumulates because it is metabolically stable against S1P lyase. Therefore, in the present investigation we have analysed whether cis-4M-Sph has an influence on G-protein-coupled S1P receptors. We showed that incubation with cis-4M-Sph $\leq$1 μM leads to internalization of G-protein-coupled S1P receptors and desensitization of S1P-induced $[Ca^{2+}]_i$ increases, and that these effects are dependent on expression of SphKs. We furthermore demonstrate that cis-4M-S1P accumulates in the supernatants of HEK-293 cells incubated with cis-4M-Sph. A model is proposed in which the expression of transport mechanisms and phosphorylation/dephosphorylation equilibria determine the net cellular response to sphingosine analogues.

2. Materials and methods

2.1. Materials

Lysophosphatidic acid (LPA), carbachol and fatty acid-free bovine serum albumin (BSA) were purchased from Sigma–Aldrich (Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany). Fura-2/AM was from Molecular Probes/Invitrogen (Invitrogen GmbH, Karlsruhe, Germany). Pertussis toxin (PTX) and ionomycin were from Calbiochem/Merck Biosciences (Merck Biosciences GmbH, Schwalbach, Germany). S1P and sphingosine were obtained from Biomol (Biomol GmbH, Hamburg, Germany). FTY720 was a kind gift from Novartis Pharma AG (Basel, Switzerland), and cis-4M-Sph was synthesised as described before [22]. Cis-4M-Sph, sphingosine and FTY720 were dissolved at 10 mM in methanol and further diluted in 1 mg/ml fatty acid-free BSA before addition to the cells. The respective solvent was used as control.

2.2. Plasmids

$S1P_1$–GFP (in pcDNA3.1) was provided by Dr. Timothy Hla (University of Connecticut Health Center, Farmington, USA). 3xHA–$S1P_2$ and 3xHA–$S1P_3$ (both in pcDNA3.1) were obtained from Missouri S&T cDNA Resource Center, Rolla, MO, USA.

2.3. Cell culture and transfection

HEK-293 cells stably expressing the M_3 muscarinic acetylcholine receptor were cultured in DMEM/F12 containing 10% fetal calf serum, 100 U/ml penicillin G and 0.1 mg/ml streptomycin in a humidified atmosphere of 5% CO_2/95% air at 37 °C. Stock cultures of HEK-293 cells were kept in the presence of 0.5 mg/ml G418. Mouse embryonic fibroblasts (MEFs) lacking SphK1 were a kind gift from Dr. Timothy Hla (University of Connecticut Health Center, Farmington, USA). MEFs lacking SphK2 were prepared from SphK2-deficient mice according to standard procedures [23]. MEFs were cultured in DMEM/F12 medium with 20% fetal calf serum, 100 U/ml penicillin G and 0.1 mg/ml streptomycin. For transfection, cells were grown in 145 mm dishes and transfected using the Ca^{2+} phosphate precipitation method (50 μg DNA per dish). Transfection efficiency was analysed by fluorescence microscopy of living or paraformaldehyde-fixed and antibody-stained cells. Before experiments, the cells were kept for 24 h in serum-free medium.

2.4. $[Ca^{2+}]_i$ measurements

Measurements of $[Ca^{2+}]_i$ were performed with fura-2 in a Hitachi F2500 spectrofluorometer as described [24]. Briefly, monolayers of HEK-293 cells or trypsinized MEFs were detached with Hank's balanced salt solution (HBSS; 118 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose and 15 mM HEPES, pH 7.4) and loaded with 1 μM fura-2/AM in HBSS for 1 h at 37 °C. Thereafter, the cells were washed with HBSS, resuspended at a density of $\sim 1 \times 10^6$ cells/ml, and used for $[Ca^{2+}]_i$ measurements at room temperature within the next hour. For determination of $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}, the cells were suspended in Ca^{2+}-free HBSS, 50 μM EGTA was added shortly before stimulation with agonists, and 1 mM $CaCl_2$ was re-added before determination of maximum and minimum fluorescence.

2.5. Immunocytochemistry and fluorescence imaging

HEK-293 cells transfected with HA-tagged $S1P_2$ or $S1P_3$ receptors were seeded onto poly-L-lysine-coated glass coverslips, fixed for 1 h with 4% paraformaldehyde on ice and permeabilized with ice-cold methanol for 4 min. After treatment with 5% milk powder for 1 h, the cells were incubated with an anti-HA antibody (clone 12CA5, Roche Applied Science, Mannheim, Germany) followed by a goat anti-mouse IgG/Alexa Fluor 488-conjugate antibody (Invitrogen GmbH, Karlsruhe, Germany) according to the manufacturer's recommendations. Images were obtained by confocal laser scanning microscopy using a Zeiss LSM510 inverted confocal laser scanning microscope and a Plan-Apochromat 63×/1.4 oil immersion objective (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). GFP and Alexa Fluor 488 were excited with the 488 nm line of an argon laser, while emission was recorded with a 505 nm long pass filter.

2.6. Lipid extraction and liquid chromatography–tandem mass spectrometry (LC–MS/MS)

HEK-293 cells seeded onto 35 mm dishes were grown to near confluence and treated with cis-4M-Sph, FTY720 or vehicle in serum-free medium for 16 h before lipid extraction. Supernatants were collected, centrifuged to remove cellular debris and transferred to glass tubes. 1 ml methanol supplemented with internal standard lipids (D-*erythro*-C17-ceramide, D-*erythro*-C17-sphingosine, D-*erythro*-C17-S1P; Avanti Polar Lipids Inc., Alabaster, AL, USA), 140 μl 1 N HCl and 400 μl salt solution (7.4% KCl, 0.4% $CaCl_2$, 0.34% $MgCl_2$) were added. Lipids were extracted three times with chloroform. The combined chloroform phases were dried on speed-vacuum. Cell pellets were incubated with 1 ml methanol including the internal standards and 35 μl 1 N HCl for 1 h on ice. Thereafter, the pellets were scraped off the cell culture dishes and transferred to glass tubes. After washing the dishes once with 1.2 ml methanol, 1.6 ml salt solution (0.74% KCl, 0.04% $CaCl_2$, 0.034% $MgCl_2$) was added to the combined methanol phases, and lipids were extracted three times with chloroform. The combined chloroform phases were dried on speed-vacuum. All dried samples were dissolved in DMSO with 2% HCl and analysed by LC–MS/MS essentially as described before [25]. A hybrid triple quadrupole/linear ion trap mass spectrometer 4000 Q TRAP equipped with a Turbo V ion spray source (Applied Biosystems, Darmstadt, Germany) operating in positive electrospray ionisation mode was used for detection. Chromatographic separations were performed by HPLC with a Luna C18(2) reversed phase column (150 mm × 2 mm, 5 μm particle size and 100 Å pore size) (Phenomenex, Aschaffenburg, Germany). 10 μl of the extracted samples were injected. The mobile phase consisted of eluent A (0.1% formic acid) and eluent B (0.1% formic acid in acetonitrile/tetrahydrofuran 50/50). At a flow rate of 0.3 ml/min, A:B was kept at 57.5:42.5 for 0.6 min, then increased linearly to 0:100 within 4.4 min, after 5 min decreased linearly back to 57.5:42.5 within 0.5 min, and kept constant until end of runtime after 14 min. Sphingosine, S1P, cis-4M-Sph and cis-4M-S1P eluted after 6.33 ± 0.03 min ($n = 29$), 6.91 ± 0.02 min ($n = 29$), 6.38 ± 0.05 min ($n = 12$) and 6.92 ± 0.02 min ($n = 9$), respectively (means ± SD). Precursor-to-product ion transitions of m/z 380.1 → 264.2 for S1P (collision energy 27 V), m/z 300.3 → 252.3 for sphingosine (26 V), m/z 314.4 → 296.2 for cis-4M-Sph (17 V), m/z 394.5 → 278.3 for cis-4M-S1P (30 V), m/z 366.2 → 250.1 for C17-S1P (23 V) and m/z 286.2 → 238.1 for C17-SPH (26 V) were used (see Fig. 4). Quantification was performed with Analyst 1.5 (Applied Biosystems, Darmstadt, Germany). Analyte peak area counts were divided by peak area counts of internal standards to calculate area ratios. Sphingosine and cis-4M-Sph were normalized to C17-sphingosine, and S1P as well as cis-4M-S1P were normalized to C17–S1P.

2.7. Data presentation and analysis

Fluorescence images are representative for at least three similar experiments. The images were edited using the program LSM Image Browser (www.zeiss.com/micro). S1P receptor internalization was quantified from the images of the GFP-tagged $S1P_1$ receptor and the HA-tagged and immunostained $S1P_2$ receptor by calculating the ratio of the cytosolic and the plasma membrane fluorescence. Data analyses, statistical tests, non-linear regression analysis of concentration–response curves and diagram presentations were performed with the Prism program (GraphPad Software, San Diego, CA, USA). Averaged data are expressed as means ± SEM from the indicated number (n) of experiments or means ± SD from a representative experiment with the indicated number (n) of replicates.

3. Results

To test our hypothesis that phosphorylated cis-4M-Sph might be able to interact with G-protein-coupled S1P receptors, we first analysed whether cis-4M-Sph was able to internalize S1P receptors in a manner similar to S1P or FTY720. As shown in Fig. 1, $S1P_1$, $S1P_2$ and $S1P_3$ receptors expressed in HEK-293 cells were localized at the plasma membrane and internalized by treatment with 1 μM S1P for 16 h. Stimulation with 1 μM cis-4M-Sph caused a strong internalization of $S1P_2$ and $S1P_3$ while $S1P_1$ was considerably less affected. A quantitative analysis comparing the internalization of $S1P_1$ and $S1P_2$ revealed that under the applied conditions the effect of cis-4M-Sph on the $S1P_1$ receptor was not significant while $S1P_2$ was strongly internalized by the compound (Fig. 1). In comparison, FTY720 induced a strong internalization of the $S1P_1$ receptor, followed by $S1P_3$, while it had no influence on the plasma membrane localisation of $S1P_2$ (Fig. 1). This is in agreement with the fact that FTY720-phosphate can activate all S1P receptors except $S1P_2$ [4,5].

Since cis-4M-Sph internalized $S1P_2$ and $S1P_3$, i.e., the receptors that mediate S1P-induced $[Ca^{2+}]_i$ increases in HEK-293 cells [26], we analysed whether cis-4M-Sph was able to desensitize Ca^{2+} signalling by S1P. Indeed, treatment of the cells with 10 μM cis-4M-Sph for 2 h fully prevented $[Ca^{2+}]_i$ increases by subsequent addition of 100 nm S1P (Fig. 2A). Sphingosine, which is similarly taken up and phosphorylated, did not desensitize S1P-induced $[Ca^{2+}]_i$ increases. Cis-4M-Sph did not inhibit $[Ca^{2+}]_i$ increases by other agonists. 10 μM cis-4M-Sph only slightly reduced $[Ca^{2+}]_i$ increases by LPA, an effect that was also observed with sphingosine (Fig. 2A). The inhibition of S1P-induced $[Ca^{2+}]_i$ increases by cis-4M-Sph was time- and concentration-dependent (Fig. 2B). When the cells were pre-treated for 5 min, 10 μM cis-4M-Sph reduced S1P-induced $[Ca^{2+}]_i$ increases by ~68%, and 2.5 μM cis-4M-Sph by ~39%, while 0.63 μM was inactive. In comparison, after incubation for 16 h, 1 μM cis-4M-Sph reduced S1P-induced $[Ca^{2+}]_i$ increases by ~68% (Fig. 2B). The concentration–response curve was shifted to the left by longer incubation periods. This effect argues against a competitive inhibition of S1P receptors by cis-4M-Sph. Since cis-4M-Sph is readily phosphorylated inside cells, we furthermore addressed the question whether cis-4M-Sph or cis-4M-S1P mediated the desensitization of S1P receptors. As shown in Fig. 2C, cis-4M-Sph reduced S1P-induced $[Ca^{2+}]_i$ increases in mouse embryonic fibroblasts deficient in either SphK1 or SphK2. However, the effect of cis-4M-Sph was significantly less pronounced in SphK1-deficient cells (49% inhibition) and SphK2-deficient cells (17% inhibition) than in wild type cells (67% inhibition). It is concluded that desensitization of S1P receptor-induced $[Ca^{2+}]_i$ increases is mediated by cis-4M-S1P, which is generated predominantly by SphK2 with a minor contribution of SphK1. FTY720, which does not affect the $S1P_2$ receptor, did not desensitize S1P-induced $[Ca^{2+}]_i$ increases and in general had no influence on $[Ca^{2+}]_i$ increases induced by various agonists in HEK-293 cells (Fig. 2D).

It has been published before that addition of 10 μM cis-4M-Sph induces $[Ca^{2+}]_i$ increases in Swiss 3T3 fibroblasts [19]. Therefore, we addressed the question whether cis-4M-Sph itself induced $[Ca^{2+}]_i$ increases before desensitizing $[Ca^{2+}]_i$ increases by S1P. Indeed, as shown in Fig. 3A, 10 μM cis-4M-Sph induced $[Ca^{2+}]_i$ increases in HEK-293 cells that developed more slowly than those induced by a maximally active concentration of S1P, but significantly faster than those by 10 μM sphingosine. $[Ca^{2+}]_i$ increases by cis-4M-Sph were nearly fully due to mobilization of Ca^{2+} from intracellular stores (Fig. 3B). 10 μM of cis-4M-Sph were required for significant increases in $[Ca^{2+}]_i$; 3 μM caused only small $[Ca^{2+}]_i$ increases (Fig. 3C) whereas concentrations below 1 μM were inactive. Thus, at a concentration that was able to largely prevent $[Ca^{2+}]_i$ increases by S1P, cis-4M-Sph itself did not

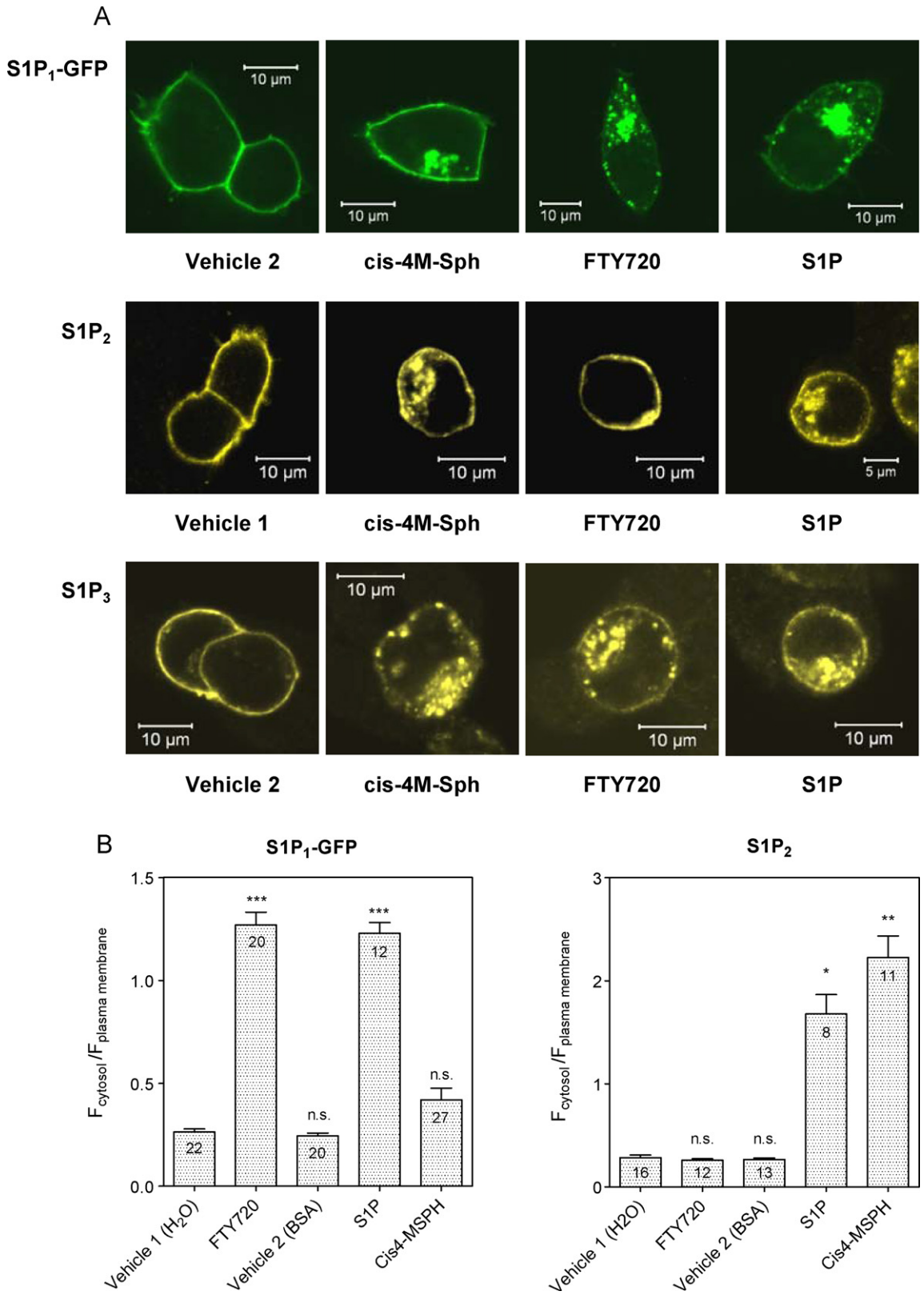

Fig. 1. Internalization of S1P receptors by cis-4M-Sph. The GFP-tagged $S1P_1$ receptor and HA-tagged $S1P_2$ and $S1P_3$ receptors were expressed in HEK-293 cells and stimulated with or without 1 μM of cis-4M-Sph, S1P or FTY720 for 16 h. $S1P_2$ and $S1P_3$ were immunostained with anti-HA and goat anti-mouse IgG/Alexa Fluor 488-conjugate antibody. Visualization was performed by confocal microscopy. (A) Representative images. There was no difference in the appearance between cells treated with vehicle 1 (H_2O; vehicle for FTY720) or vehicle 2 (0.1 mg/ml BSA in H_2O; vehicle for S1P and cis-4M-Sph). (B) Internalization of the $S1P_1$ and $S1P_2$ receptors was performed by calculating the ratio of the cytosolic and the plasma membrane fluorescence. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; n.s., not significant in one-way ANOVA comparing FTY720 and vehicle 2 to vehicle 1, and S1P and cis-4M-Sph to vehicle 2, respectively. The figures given on the bars represent the number of cells that were analysed.

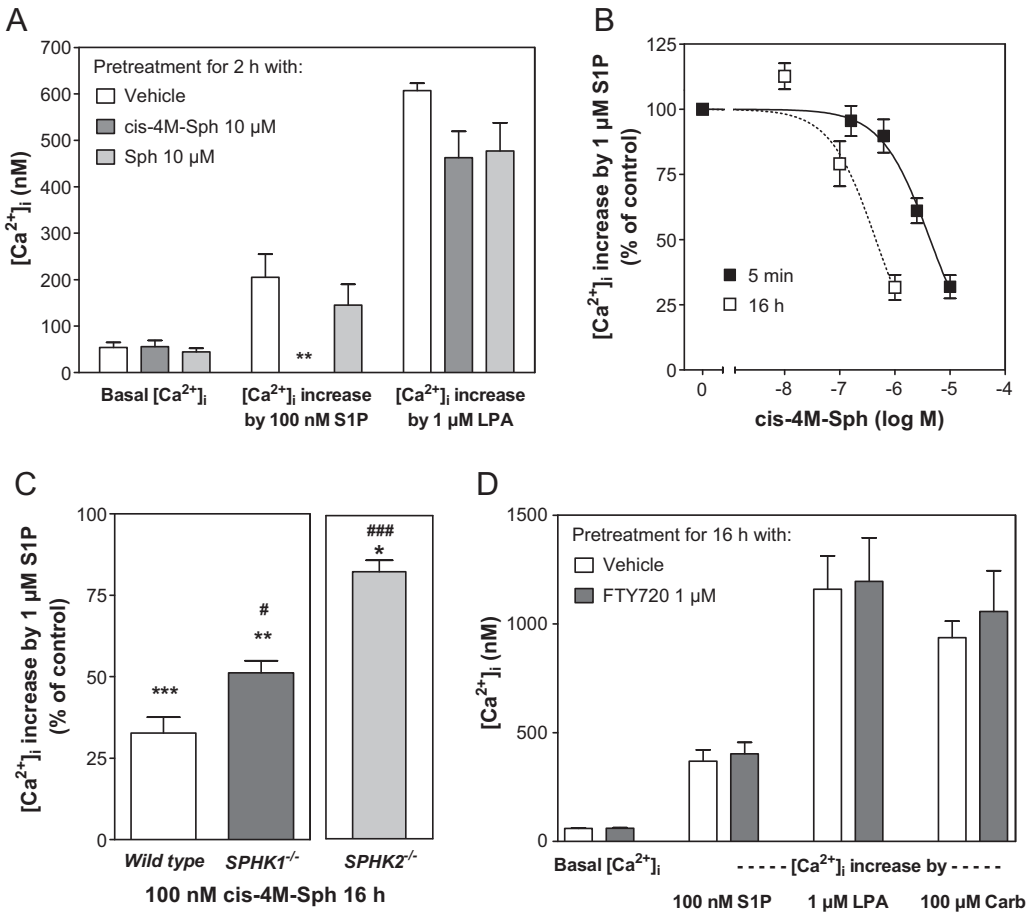

Fig. 2. Desensitization of S1P-induced $[Ca^{2+}]_i$ increases by cis-4M-Sph. (A) influence of cis-4M-Sph on basal $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ increases induced by S1P or LPA in HEK-293 cells. The cells were pretreated for 2 h with 10 μM cis-4M-Sph, 10 μM sphingosine or vehicle before measurements. The values represent means ± SD of a representative experiment ($n = 3$). $^{**}p < 0.01$ in one-way ANOVA comparing S1P-induced $[Ca^{2+}]_i$ increases in the absence or presence of cis-4M-Sph or Sph. (B) Concentration- and time-dependence of cis-4M-Sph-mediated desensitization of S1P-induced $[Ca^{2+}]_i$ increases in HEK-293-cells. The cells were pre-incubated for 5 min or 16 h with the indicated concentrations of cis-4M-Sph before $[Ca^{2+}]_i$ increases by 1 μM S1P were measured. The data are expressed as % of vehicle-treated cells and represent means ± SEM of 5 (5 min) or 3 (16 h) independent experiments. (C) Dependence of cis-4M-Sph-induced desensitization on sphingosine kinases. Mouse embryonic fibroblasts from wild type, SphK1-deficient (*SPHK1*$^{-/-}$) or SphK2-deficient (*SPHK2*$^{-/-}$) mice were treated with 100 nm cis-4M-Sph or vehicle for 16 h before measurement of S1P-induced $[Ca^{2+}]_i$ increases. The values are expressed as % of vehicle-treated cells and represent means ± SEM of 4 experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; cis-4M-Sph-treated cells compared to vehicle-treated cells in one-sample-*t*-test. $\#p < 0.05$; $\#\#\#p < 0.001$; SphK-deficient cells compared to wild type cells in one-way ANOVA. (D) Influence of FTY720 on basal $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ increases induced by S1P, LPA or carbachol (Carb) in HEK-293-cells. The cells were pretreated for 16 h with 1 μM FTY720 or vehicle before measurements. The values represent means ± SEM of 6 independent experiments.

increase $[Ca^{2+}]_i$ to a significant extent, suggesting that the desensitization process occurred in a slow progressing manner. Again we analysed whether cis-4M-Sph or eventually cis-4M-S1P induced the $[Ca^{2+}]_i$ increases. As shown in Fig. 3D and E, $[Ca^{2+}]_i$ increases by 10 μM cis-4M-Sph were small and developed very slowly in SphK1-deficient MEFs, while they were apparently not affected by deletion of SphK2. It is concluded that acute increases in $[Ca^{2+}]_i$ induced by cis-4M-Sph are mediated by cis-4M-S1P that is generated predominantly by SphK1. Thus, the two effects of cis-4M-Sph, i.e., induction of acute $[Ca^{2+}]_i$ increases on one hand and desensitization of S1P-induced $[Ca^{2+}]_i$ increases on the other hand, are mediated by different pools of cis-4M-S1P, one generated largely by SphK1 and the other generated predominantly by SphK2. Finally we wondered whether acute $[Ca^{2+}]_i$ increases by cis-4M-Sph were due to activation of G-protein-coupled S1P receptors. Although it is well known that S1P-induced $[Ca^{2+}]_i$ increases in HEK-293 cells are fully blocked by PTX and thus mediated by G_i-coupled receptors [12], $[Ca^{2+}]_i$ increases by cis-4M-Sph were not at all affected by PTX (Fig. 3F) and thus not mediated by G-protein-coupled S1P receptors in these cells.

The above described internalization and desensitization of S1P receptors requires the extracellular occurrence of cis-4M-S1P, which has not been described so far. Therefore, we analysed cis-4M-Sph and cis-4M-S1P in pellets and supernatants of HEK-293 cells that had been incubated for ~16 h with 1 μM or 10 μM of cis-4M-Sph. As shown in Fig. 4A, cis-4M-Sph and cis-4M-S1P were detected by LC–MS/MS with precursor-to-product ion transitions of m/z 314.4 → 296.2 for cis-4M-Sph and m/z 394.5 → 278.3 for cis-4M-S1P. The measurements were performed in the presence of 1 mM sodium orthovanadate to prevent dephosphorylation of cis-4M-S1P by ectophosphatases. In the presence of orthovanadate, cellular levels of sphingosine and S1P amounted to 7.3 ± 0.5 ng/mg and 3.5 ± 0.6 ng/mg protein, respectively (means ± SD of tripletts), whereas 9.6 ± 1.9 ng/mg and 0.50 ± 0.05 ng/mg protein of sphingosine and S1P, respectively, were measured in untreated cells (means ± SEM, $n = 3$ experiments). Thus, cellular S1P levels in particular were elevated by the treatment. Since cis-4M-S1P for generation of a standard curve was not available, all measurements of cis-4M-Sph and cis-4M-S1P were evaluated by comparing peak area counts of these lipids, after normalization to internal standards, with sphingosine and S1P. After 16 h of incubation with 1 μM cis-4M-Sph, cellular levels of cis-4M-Sph were ~50-fold higher than those of sphingosine and levels of cis-4M-S1P amounted to ~62% of sphingosine, respectively (Fig. 4B). After incubation with 10 μM

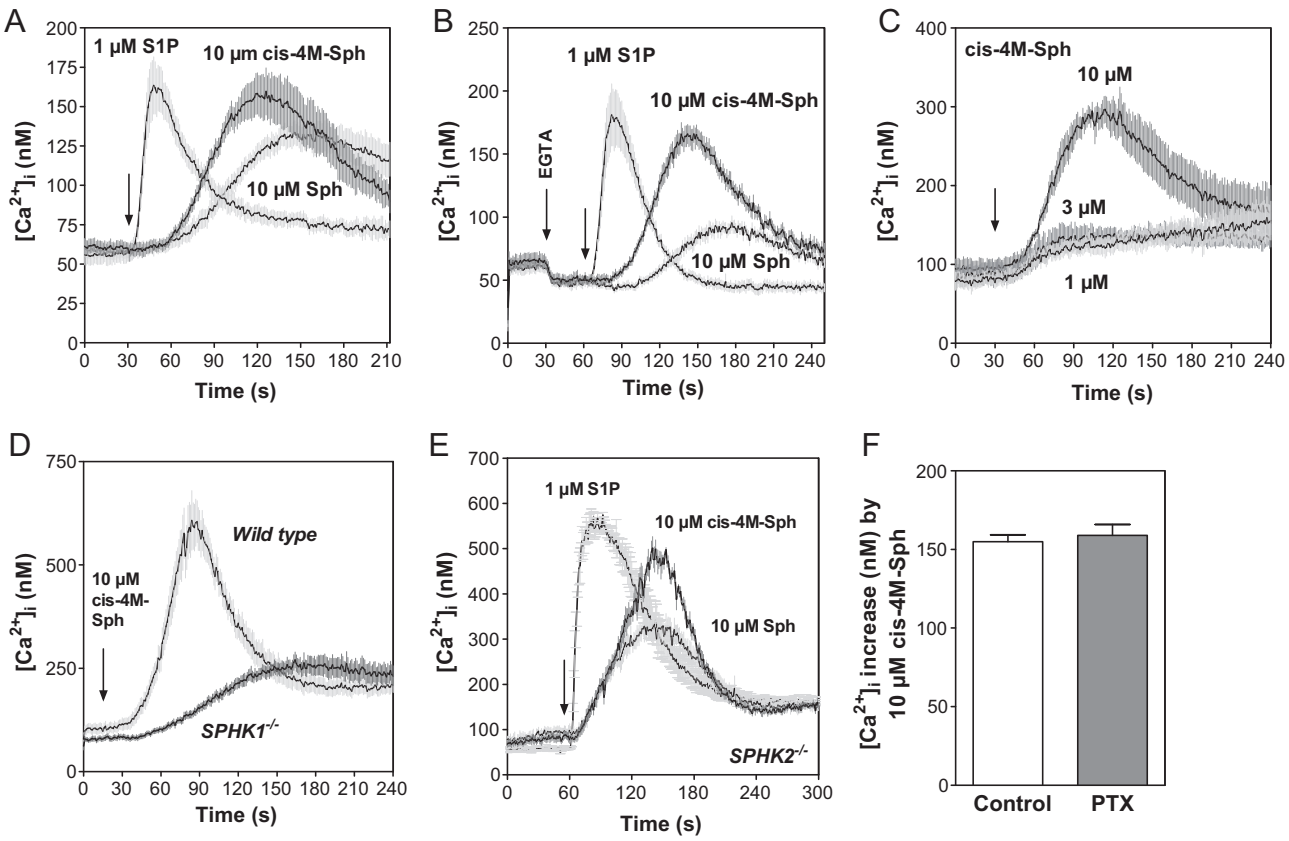

Fig. 3. Induction of acute $[Ca^{2+}]_i$ increases by cis-4M-Sph. (A) Time courses of $[Ca^{2+}]_i$ in the presence of 1 mM extracellular Ca^{2+}. HEK-293 cells were stimulated with cis-4M-Sph ($n = 9$), S1P ($n = 11$), or sphingosine ($n = 11$) at the indicated concentrations (means ± SEM). (B) Time courses of $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. HEK-293 cells were resuspended in Ca^{2+}-free buffer and 50 µM EGTA was added ~30 s before stimulation with cis-4M-Sph, S1P, or sphingosine at the indicated concentrations (means ± SEM, $n = 12$ each). (C) Concentration dependence of cis-4M-Sph-induced $[Ca^{2+}]_i$ increases. HEK-293 cells were stimulated with 10 µM ($n = 6$), 3 µM ($n = 5$) or 1 µM ($n = 4$) cis-4M-Sph (means ± SEM). (D and E) Role of sphingosine kinases in cis-4M-Sph-induced $[Ca^{2+}]_i$ increases. (D) Cis-4M-Sph-induced $[Ca^{2+}]_i$-increases in wild type and *SPHK1*$^{-/-}$-MEFs (means ± SEM, $n = 16$ (wild type) or 17 (*SPHK1*$^{-/-}$)). (E) Time courses of $[Ca^{2+}]_i$ in *SPHK2*$^{-/-}$-MEFs stimulated with cis-4M-Sph, S1P or sphingosine at the indicated concentrations (means ± SD, $n = 3$ each). (F) Influence of PTX on $[Ca^{2+}]_i$ increases induced by 10 µM cis-4M-Sph in HEK-293 cells. The cells were pretreated without or with 100 ng/ml PTX for 16 h (means ± SEM, $n = 4$).

cis-4M-Sph, cellular levels of cis-4M-Sph and cis-4M-S1P were ~270-fold and ~15-fold higher than those of sphingosine (Fig. 4B). Thus, both lipids accumulated inside the cells. Although neither sphingosine nor S1P were consistently measurable in supernatants of HEK-293 cells, cis-4M-Sph and cis-4M-S1P were both detected in the supernatants of treated cells. As shown in Fig. 4C, 2.4% of cellular cis-4M-Sph and 7.1% of cis-4M-S1P were detected in the media of cells treated with 1 µM cis-4M-Sph. After treatment with 10 µM of cis-4M-Sph, 8.4% and 31.0% of cellular cis-4M-Sph and cis-4M-S1P were detected in the supernatants. Thus, particularly cis-4M-S1P accumulated in the media. Similar experiments with FTY720, however in the absence of orthovanadate, resulted in 27.0 ± 5.4 ng/mg and 2.18 ± 0.39 ng/mg protein of FTY720 and FTY720-phosphate in HEK-293 cell pellets, corresponding to ~280% and ~23% of sphingosine, respectively. 5.7 ± 0.2% of FTY720 and 4.5 ± 2.5% of FTY720-phosphate appeared in the media, respectively, after 16 h of incubation with 1 µM FTY720 (means ± SEM, $n = 3$ experiments). These results show that the concentrations of cis-4M-S1P in the supernatants were at least of the same magnitude as those of FTY720-phosphate, suggesting that there was enough cis-4M-S1P in the media for inducing the observed internalization of G-protein-coupled S1P receptors.

4. Discussion

The sphingosine derivative, cis-4M-Sph, has been described as a compound that is readily taken up by cells and phosphorylated to cis-4M-S1P which accumulates intracellularly and, at a concentration of 10 µM, exerts opposing effects on neuronal cells (i.e., apoptosis [18]) and fibroblasts (i.e., promotion of cell growth [19]). Here, for the first time a possible interaction of cis-4M-Sph with G-protein-coupled S1P receptors has been considered and effects of cis-4M-Sph have been observed at concentrations below 10 µM. We observed that cis-4M-Sph caused an internalization of S1P receptors and a desensitization of S1P-induced $[Ca^{2+}]_i$ increases in a time- and concentration-dependent manner. It is highly likely that the internalization and desensitization is a consequence of an agonistic activity of cis-4M-S1P at S1P receptors for the following reasons: (1) There was specificity so that $[Ca^{2+}]_i$ increases by agonists other than S1P were not affected to a similar degree, which would be the case if cis-4M-Sph acted for example as an activator of protein kinases C that can lead to receptor desensitization and shutdown of Ca^{2+} signalling. (2) The expression of SphKs was required for this effect, indicating that it was mediated by cis-4M-S1P. (3) Cis-4M-S1P indeed could be detected in the supernatants of HEK-293 cells incubated with cis-4M-Sph, which indicates a transport of cis-4M-S1P across the plasma membrane since SphKs are located intracellularly. In agreement with a previous report with Swiss 3T3 fibroblasts, cis-4M-Sph at 10 µM induced $[Ca^{2+}]_i$ increases in HEK-293 cells. This effect was due to mobilization of stored Ca^{2+} and not observed at lower concentrations of the compound. We think that this activity of cis-4M-Sph is not related to internalization and desensitization of G-protein-coupled S1P receptors since it was independent of G_i proteins,

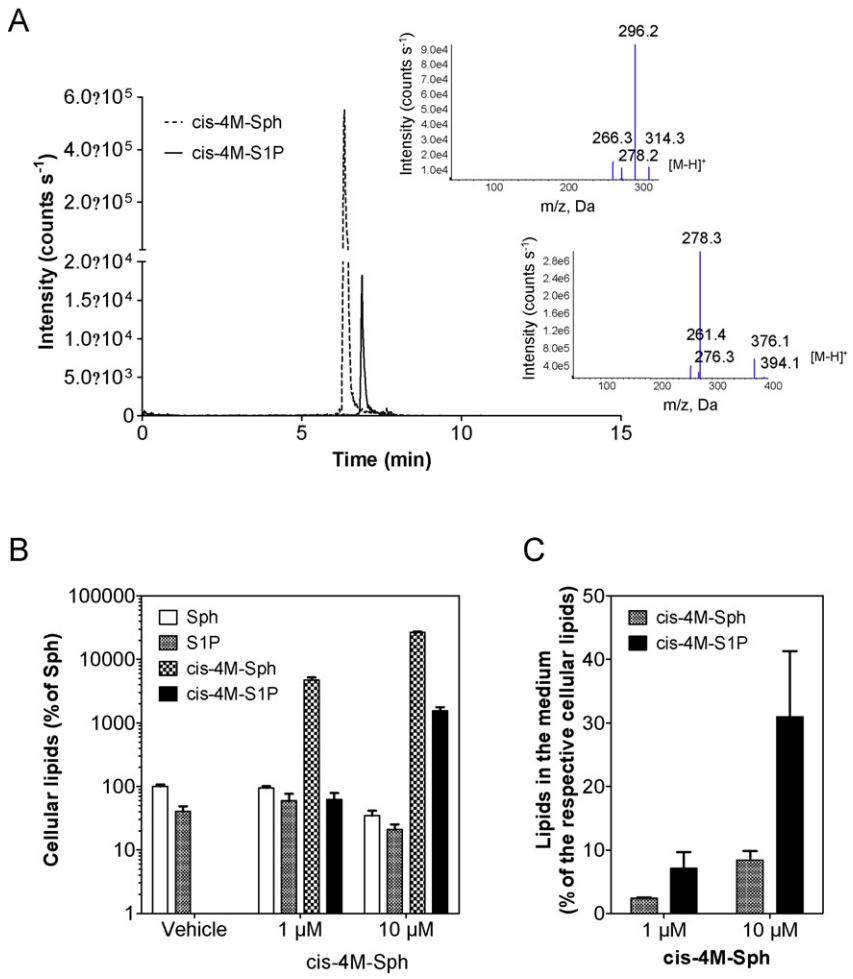

Fig. 4. Detection of cis-4M-S1P in the supernatants of HEK-293 cells by LC–MS/MS. (A) Representative chromatogram of the extracted supernatant of cells that had been treated with 10 μM cis-4M-Sph for 16 h in the presence of 1 mM sodium orthovanadate. Inserts, enhanced product ion spectra of cis-4M-Sph and cis-4M-S1P (collision energy 35 ± 15 V). (B) Cellular levels of sphingosine, S1P, cis-4M-Sph and cis-4M-S1P in cells treated for 16 h with vehicle, 1 μM or 10 μM of cis-4M-Sph in the presence of 1 mM sodium orthovanadate. Peak area counts of the lipids were normalized to peak area counts of the respective internal standards and are expressed as % of sphingosine (means ± SD of triplets). (C) Lipid levels in the supernatants of cells treated for 16 h with 1 μM or 10 μM of cis-4M-Sph. Peak area counts of cis-4M-Sph and cis-4M-S1P in the medium were normalized to peak area counts of internal standards and are expressed as % of the respective area ratios within the cell pellets (means ± SD of triplets).

which otherwise mediate S1P-induced $[Ca^{2+}]_i$ increases in these cells [12], and caused by a distinct pool of cis-4M-S1P. The data show that S1P receptor desensitization was strongly dependent on expression of SphK2, while Ca^{2+} mobilization rather required SphK1. Although S1P receptor desensitization by cis-4M-Sph was small in MEFs lacking SphK2, it was nevertheless significant, indicating that a minor fraction of cis-4M-S1P produced by SphK1 was exported and able to interact with the plasma membrane receptors. Herein it might be important that SphK1 was upregulated in SphK2-deficient MEFs by about 2–3-fold as measured by quantitative PCR (data not shown). It is concluded that the cis-4M-S1P that was produced by SphK1 mobilized Ca^{2+} directly from the stores as previously demonstrated for S1P [12], and that relatively high concentrations of cis-4M-Sph are required for this effect. However, another mechanism, for example activation not of S1P receptors or G_i-coupled receptors but other plasma membrane receptors, cannot be fully excluded.

The measurements of cis-4M-Sph and cis-4M-S1P by LC–MS/MS hampered by the fact that neither compound was commercially available and that we did not have enough cis-4M-Sph for generation of sufficient amounts of cis-4M-S1P by in vitro-phosphorylation. Therefore, peak area counts for cis-4M-Sph and cis-4M-S1P, normalized to the respective internal control, were compared to peak area counts of sphingosine and S1P. Furthermore, the measurements were performed in the presence of sodium orthovanadate to prevent the dephosphorylation of cis-4M-S1P. It has been shown before that 1 mM orthovanadate was sufficient to fully block the dephosphorylation of exogenous S1P in HTC_4 cells [27]. This treatment did not have a major influence on sphingosine levels, but elevated cellular S1P levels from ~5% to ~50% of sphingosine. Thus, orthovanadate had a considerable impact on cellular sphingosine/S1P equilibria. Comparing cis-4M-Sph and cis-4M-S1P with sphingosine and S1P, we find substantial amounts of these lipids in cell pellets and supernatants of HEK-293 cells incubated with cis-4M-Sph. As described above, after incubation with 1 μM cis-4M-Sph, cellular levels of cis-4M-S1P reached ~60% of sphingosine, and ~7% thereof were measured in the media. In comparison, after incubation with 1 μM FTY720, cellular levels of FTY720-phosphate were ~20% of sphingosine and ~5% thereof were measured in the media. Thus, although the measurements are not directly comparable because orthovanadate was used in the experiments with cis-4M-Sph, we are convinced that cis-4M-S1P in the supernatants was of roughly the same magnitude as FTY720-phosphate. However, it is a good question

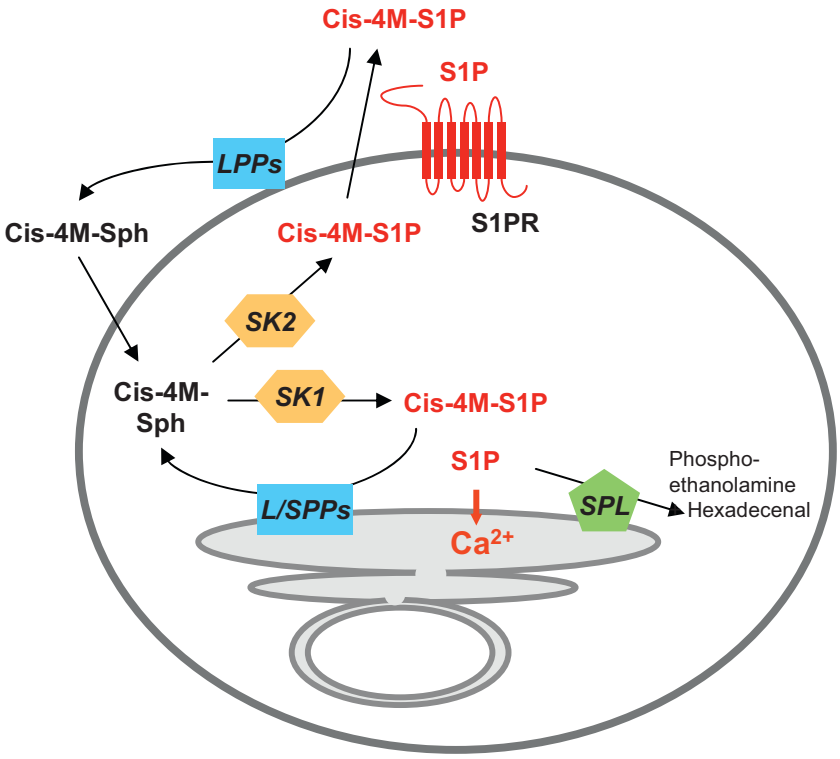

Fig. 5. Regulation of cis-4M-S1P availability at S1P receptors by phosphorylation–dephosphorylation equilibria and membrane transport mechanisms. Cis-4M-Sph is readily taken up by the cells and phosphorylated by SphK1 and SphK2. Intracellular pools of cis-4M-S1P that mobilize Ca^{2+} from the stores are largely dependent on phosphorylation by SphK1 (SK1), while pools of cis-4M-S1P that are secreted and desensitize G-protein-coupled S1P receptors are predominantly generated by SphK2 (SK2). Extra- and intracellular cis-4M-S1P is probably dephosphorylated by phosphatases such as S1P phosphatases (SPPs) or lipid phosphate phosphatases (LPPs), but stable against S1P lyase, since both cis-4M-Sph and cis-4M-S1P accumulate intracellularly and in the medium of cells exposed to cis-4M-Sph. It is highly likely that transport mechanisms are required for secretion of cis-4M-S1P as shown before for S1P.

whether the concentrations measured in the supernatants can be compared to those directly at the S1P surface receptors because it is possible that a substantial amount of the extracellular lipids sticks to the plasma membranes. The experiments furthermore show that after incubation with 10 μM cis-4M-Sph for 16 h, the cellular content of sphingosine and S1P was slightly decreased (Fig. 4B). This effect was not observed at 1 μM cis-4M-Sph and might be caused by the previously described inhibition of serine palmitoyltransferase which can occur at high concentrations of the lipid [18].

Although the stability of cis-4M-S1P against S1P lyase has not been measured directly, there is ample experimental evidence that this compound is only a poor substrate if at all of S1P lyase ([18]; see Section 1). Similarly, FTY720-phosphate is stable against S1P lyase, and FTY720 even inhibits the enzyme [28]. This implicates that the availability of the lipid phosphates at S1P surface receptors is determined by phosphorylation/dephosphorylation equilibria, and, because SphKs are intracellular enzymes, on transport mechanisms across the plasma membrane (see Fig. 5 for illustration). Lipid phosphate phosphatases or specific S1P phosphatases are proteins with transmembrane domains that reside both at intracellular membranes and at the plasma membrane, where they act as ectophosphatases (for review, see [7]). FTY720-phosphate is preferentially cleaved by LPP3 [29]. The transport of S1P across the plasma membrane can be catalysed by ABC transporters [30], or by the *SPNS2* gene product [31,32]. The mechanism by which FTY720-phosphate is extruded has not been identified so far. We therefore suggest that the response to sphingosine analogues as S1P receptor modulating prodrugs will be tissue specific, depending on the local abundance of sphingosine kinases, phosphatases and transporters (Fig. 5).

Despite all similarities, cis-4M-Sph clearly differed from FTY720 with regard to the S1P receptor profile. Thus, cis-4M-Sph desensitized S1P-induced $[Ca^{2+}]_i$ increases, an effect that was observed with FTY720 so far only in an artificial $S1P_1$ receptor overexpression system [33]. Previous studies have shown that the concentration–response curve of S1P-induced $[Ca^{2+}]_i$ increases in HEK-293 cells was shifted to the right by VPC23019 [25], which is a $S1P_{1/3}$ receptor antagonist [34,35]. S1P-induced $[Ca^{2+}]_i$ increases in HEK-293 cells were furthermore partially inhibited by JTE-013 (own unpublished data) which is a $S1P_2$ receptor antagonist [35,36]. Thus, $[Ca^{2+}]_i$ increases by S1P are mediated by the $S1P_2$ and $S1P_3$ receptor subtypes in HEK-293 cells, while $S1P_1$ has an inhibitory influence on Ca^{2+} signalling [37]. The desensitization observed with cis-4M-Sph, but not with FTY720, can therefore be traced back to the observed internalization of $S1P_2$ and $S1P_3$ receptors. Further studies of S1P receptor subtype selectivity, including K_d measurements and quantitative analyses of the activation of S1P receptor signalling pathways, are urgently needed but require the synthesis of cis-4M-S1P. In case a preference of cis-4M-S1P for $S1P_2/S1P_3$ compared to $S1P_1$ can be confirmed, it may be used as a lead compound for synthesis of $S1P_2/S1P_3$ receptor specific agonists/functional antagonists. As discussed above, the net response of a certain tissue to cis-4M-Sph, or related compounds, will be dependent on transport mechanisms and phosphorylation/dephosphorylation equilibria. A desensitization of S1P-induced $[Ca^{2+}]_i$ increases might be helpful to counteract S1P-induced vascular or bronchial hypercontractility [38].

Acknowledgments

We thank Claudia Häusler and Kerstin Liphardt for expert technical assistance. Furthermore, we are indebted to Svetlana Bubnova for invaluable help with LC–MS/MS sample acquisition and data evaluation, and to Luise Reinsberg for preparation and

cultivation of SphK2-deficient MEFs. This work was supported by the Deutsche Forschungsgemeinschaft (ME1734/3-1, FOG784) and the LOEWE Lipid Signalling Forschungszentrum Frankfurt (LiFF).

References

[1] Brinkmann V. FTY720 (fingolimod) in multiple sclerosis: therapeutic effects in the immune and the central nervous system. Br J Pharmacol 2009;158:1173–82.
[2] Cohen JA, Barkhof F, Comi G, Hartung HP, Khatri BO, Montalban X, et al. Oral fingolimod or intramuscular interferon for relapsing multiple sclerosis. N Engl J Med 2010;362:402–15.
[3] Kappos L, Radue EW, O'Connor P, Polman C, Hohlfeld R, Calabresi P, et al. A placebo-controlled trial of oral fingolimod in relapsing multiple sclerosis. N Engl J Med 2010;362:387–401.
[4] Brinkmann V, Davis MD, Heise CE, Albert R, Cottens S, Hof R, et al. The immune modulator FTY720 targets sphingosine-1-phosphate receptors. J Biol Chem 2002;277:21453–7.
[5] Mandala S, Hajdu R, Bergstrom J, Quackenbush E, Xie J, Milligan J, et al. Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. Science 2002;296:346–9.
[6] Alemany R, van Koppen CJ, Danneberg K, ter Braak M, Meyer zu Heringdorf D. Regulation and functional roles of sphingosine kinases. Naunyn-Schmiedeberg's Arch Pharmacol 2007;374:413–28.
[7] Meyer zu Heringdorf D, Jakobs KH. Lysophospholipid receptors: signalling, pharmacology and regulation by lysophospholipid metabolism. Biochim Biophys Acta 2007;1768:923–40.
[8] Pyne S, Lee SC, Long J, Pyne NJ. Role of sphingosine kinases and lipid phosphate phosphatases in regulating spatial sphingosine 1-phosphate signalling in health and disease. Cell Signal 2009;21:14–21.
[9] Brinkmann V. Sphingosine-1-phosphate receptors in health and disease: mechanistic insights from gene deletion studies and reverse pharmacology. Pharmacol Ther 2007;115:84–105.
[10] Choi JW, Lee CW, Chun J. Biological roles of lysophospholipid receptors revealed by genetic null mice: an update. Biochim Biophys Acta 2008;1781:531–9.
[11] Hait NC, Allegood J, Maceyka M, Strub GM, Harikumar KB, Singh SK, et al. Regulation of histone acetylation in the nucleus by sphingosine-1-phosphate. Science 2009;325:1254–7.
[12] Meyer zu Heringdorf D, Liliom K, Schaefer M, Danneberg K, Jaggar JH, Tigyi G, et al. Photolysis of intracellular caged sphingosine-1-phosphate causes Ca^{2+} mobilization independently of G-protein-coupled receptors. FEBS Lett 2003;554:443–9.
[13] Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, Brinkmann V, et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature 2004;427:355–60.
[14] Lo CG, Xu Y, Proia RL, Cyster JG. Cyclical modulation of sphingosine-1-phosphate receptor 1 surface expression during lymphocyte recirculation and relationship to lymphoid organ transit. J Exp Med 2005;201:291–301.
[15] Schwab SR, Pereira JP, Matloubian M, Xu Y, Huang Y, Cyster JG. Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. Science 2005;309:1735–9.
[16] Pappu R, Schwab SR, Cornelissen I, Pereira JP, Regard JB, Xu Y, et al. Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. Science 2007;316:295–8.
[17] Foss Jr FW, Mathews TP, Kharel Y, Kennedy PC, Snyder AH, Davis MD, et al. Synthesis and biological evaluation of sphingosine kinase substrates as sphingosine-1-phosphate receptor prodrugs. Bioorg Med Chem 2009;17:6123–36.
[18] van Echten-Deckert G, Zschoche A, Bär T, Schmidt RR, Raths A, Heinemann T, et al. Cis-4-methylsphingosine decreases sphingolipid biosynthesis by specifically interfering with serine palmitoyltransferase activity in primary cultured neurons. J Biol Chem 1997;272:15825–33.
[19] van Echten-Deckert G, Schick A, Heinemann T, Schnieders B. Phosphorylated cis-4-methylsphingosine mimics the mitogenic effect of sphingosine-1-phosphate in Swiss 3T3 fibroblasts. J Biol Chem 1998;273:23585–9.
[20] Naetzker S, Heinemann T, Figueroa-Perez S, Schnieders B, Schmidt RR, Sandhoff K, et al. Cis-4-methylsphingosine phosphate induces apoptosis in neuroblastoma cells by opposite effects on p38 and ERK mitogen-activated protein kinases. Biol Chem 2002;383:1885–94.
[21] Hagen N, van Veldhoven PP, Proia RL, Park H, Merrill Jr AH, Echten-Deckert G. Subcellular origin of sphingosine 1-phosphate is essential for its toxic effect in lyase-deficient neurons. J Biol Chem 2009;284:11346–53.
[22] Bär T, Kratzer B, Wilda R, Sandhoff K, Schmidt RR. Synthesis of methyl-branched sphingosines. Liebig's Ann Chem 1993;1993:419–26.
[23] Xu J. Preparation, culture, and immortalization of mouse embryonic fibroblasts. Curr Protoc Mol Biol 2005. doi: 10.1002/0471142727.mb2801s70. www.currentprotocols.com/protocol/mb2801.
[24] Claas RF, Ter Braak M, Hegen B, Hardel V, Angioni C, Schmidt H, et al. Enhanced Ca^{2+} storage in sphingosine-1-phosphate lyase-deficient fibroblasts. Cell Signal 2010;22:476–83.
[25] Schmidt H, Schmidt R, Geisslinger G. LC–MS/MS-analysis of sphingosine-1-phosphate and related compounds in plasma samples. Prostaglandins Other Lipid Mediat 2006;81:162–70.
[26] Lichte K, Rossi R, Danneberg K, Ter Braak M, Kürschner U, Jakobs KH, et al. Lysophospholipid receptor-mediated Ca^{2+} signaling in human keratinocytes. J Invest Dermatol 2008;128:1487–98.
[27] Peest U, Sensken SC, Andreani P, Hanel P, van Veldhoven PP, Gräler MH. S1P-lyase independent clearance of extracellular sphingosine 1-phosphate after dephosphorylation and cellular uptake. J Cell Biochem 2008;104:756–72.
[28] Bandhuvula P, Tam YY, Oskouian B, Saba JD. The immune modulator FTY720 inhibits sphingosine-1-phosphate lyase activity. J Biol Chem 2005;280:33697–700.
[29] Mechtcheriakova D, Wlachos A, Sobanov J, Bornancin F, Zlabinger G, Baumruker T, et al. FTY720-phosphate is dephosphorylated by lipid phosphate phosphatase 3. FEBS Lett 2007;581:3063–8.
[30] Kim RH, Takabe K, Milstien S, Spiegel S. Export and functions of sphingosine-1-phosphate. Biochim Biophys Acta 2009;1791:692–6.
[31] Osborne N, Brand-Arzamendi K, Ober EA, Jin SW, Verkade H, Holtzman NG, et al. The spinster homolog, two of hearts, is required for sphingosine 1-phosphate signaling in zebrafish. Curr Biol 2008;18:1882–8.
[32] Kawahara A, Nishi T, Hisano Y, Fukui H, Yamaguchi A, Mochizuki N. The sphingolipid transporter spns2 functions in migration of zebrafish myocardial precursors. Science 2009;323:524–7.
[33] Müllershausen F, Zecri F, Cetin C, Billich A, Guerini D, Seuwen K. Persistent signaling induced by FTY720-phosphate is mediated by internalized $S1P_1$ receptors. Nat Chem Biol 2009;5:428–34.
[34] Davis MD, Clemens JJ, Macdonald TL, Lynch KR. Sphingosine 1-phosphate analogs as receptor antagonists. J Biol Chem 2005;280:9833–41.
[35] Huwiler A, Pfeilschifter J. New players on the center stage: sphingosine 1-phosphate and its receptors as drug targets. Biochem Pharmacol 2008;75:1893–900.
[36] Osada M, Yatomi Y, Ohmori T, Ikeda H, Ozaki Y. Enhancement of sphingosine 1-phosphate-induced migration of vascular endothelial cells and smooth muscle cells by an EDG-5 antagonist. Biochem Biophys Res Commun 2002;299:483–7.
[37] Meyer zu Heringdorf D, Vincent ME, Lipinski M, Danneberg K, Stropp U, Wang D, et al. Inhibition of Ca^{2+} signalling by the sphingosine 1-phosphate receptor $S1P_1$. Cell Signal 2003;15:677–87.
[38] Skoura A, Hla T. Regulation of vascular physiology and pathology by the $S1P_2$ receptor subtype. Cardiovasc Res 2009;82:221–8.