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Phosphorylated FTY720 promotes astrocyte migration through sphingosine-1-phosphate receptors

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Abstract

Sphingosine-1-phosphate (S1P) receptors are widely expressed in the central nervous system where they are thought to regulate glia cell function. The phosphorylated version of fingolimod/FTY720 (FTY720P) is active on a broad spectrum of S1P receptors and the parent compound is currently in phase III clinical trials for the treatment of multiple sclerosis. Here, we aimed to identify which cell type(s) and S1P receptor(s) of the central nervous system are targeted by FTY720P. Using calcium imaging in mixed cultures from embryonic rat cortex we show that astrocytes are the major cell type responsive to FTY720P in this assay. In enriched astrocyte cultures, we detect expression of S1P1 and S1P3 receptors and demonstrate that FTY720P activates Gi protein-mediated signaling cascades. We also show that FTY720P as well as the S1P1-selective agonist SEW2871 stimulate astrocyte migration. The data indicate that FTY720P exerts its effects on astrocytes predominantly via the activation of S1P1 receptors, whereas S1P signals through both S1P1 and S1P3 receptors. We suggest that this distinct pharmacological profile of FTY720P, compared with S1P, could play a role in the therapeutic effects of FTY720 in multiple sclerosis.

Keywords: G protein-coupled receptors, multiple sclerosis, signal transduction, sphingosine-1-phosphate.
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The lipid messenger sphingosine-1-phosphate (S1P) is generated by phosphorylation of sphingosine by sphingosine kinases (Baumruker *et al.* 2005). Platelets and endothelial cells are sources of S1P, which is present at concentrations above 100 nmol/L in the plasma (Pyne and Pyne 2000). Most of the effects of S1P are mediated by a family of G protein-coupled receptors designated S1P1–S1P5, which were formerly known as products of 'endothelial differentiation genes' (Chun *et al.* 2002). S1P receptors (S1P-Rs) are targeted by the drug fingolimod (FTY720) and its immunomodulatory activity shows efficacy in animal models of transplantation (Brinkmann and Lynch 2002). More importantly, FTY720 is currently in phase III clinical trials for the treatment of the autoimmune disorder multiple sclerosis (MS) (Kappos *et al.* 2005, 2006; Gardell *et al.* 2006).

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Abbreviations used: AC, adenylyl cyclase; CHO, Chinese Hamster Ovary; FTY720, fingolimod; FTY720P, phosphorylated version of FTY720; GFAP, glial fibrillary acidic protein; IP, inositol-phophates; HBSS, Hepes-buffered saline solution; MS, multiple sclerosis; PLC, phospholipase C; PTX, pertussis toxin; S1P, sphingosine-1-phosphate; SEW2871, [5-(4-phenyl-5-trifluoromethylthiophen-2-yl)-3-(3-trifluoromethylphenyl)-(1,2,4)-oxadiazole].

Like sphingosine, FTY720 is phosphorylated *in vivo* by sphingosine kinases to yield the active metabolite FTY720-phosphate (FTY720P), which is an agonist for S1P1, 3, 4, and 5 and inactive on S1P2 receptors (Brinkmann *et al.* 2002). Treatment with FTY720 inhibits lymphocyte egress from secondary lymphoid organs. This immunomodulatory action does not result in generalized immunosuppression but rather prevents the migration of lymphocytes to the peripheral sites of inflammation (Pinschewer *et al.* 2000). The inhibition of egress from lymphoid organs is mediated by activation of S1P1 receptors on lymphocytes and is considered to underlie most of the beneficial effects of FTY720 in the treatment of MS (Mandala *et al.* 2002; Graler and Goetzl 2004; Matloubian *et al.* 2004).

As S1P receptors are ubiquitously expressed, including the CNS (Chun *et al.* 2000), the efficacy of FTY720 in MS may involve mechanisms in addition to lymphocyte trafficking. Moreover, the potencies and efficacies of FTY720P on the family of S1P receptors are different from S1P and may trigger distinct signaling events. For example, previous studies have shown that S1P regulates cell migration via S1P1 and S1P2 receptors (Okamoto *et al.* 2000), whereas the effects of FTY720P are independent of S1P2 receptors. We therefore aimed to characterize the specific effects of FTY720P on cells of the CNS. Here, we demonstrate that FTY720P triggers numerous intracellular signaling events in astrocytes mainly via the activation of S1P1 receptors. Functionally, we also show that FTY720P and a selective S1P1 receptor agonist promote astrocyte migration. Taken together, these data support the notion that FTY720P acts beyond immunomodulation and directly regulates astrocyte function.

Materials and methods

Chemicals and tools

FTY720P is a phosphorylated version of FTY720 which is a fungal metabolite (full name, 2-amino-2-[2-(4-octylphenyl)ethyl] propane-1,3-diol) derived from ISP-1 (myriocin) and was used as a pan agonist for S1P1, 3, 4, and 5 receptor subtypes. In all experiments we used the pure active (S)-enantiomer of FTY720P which corresponds to the *in vivo* phosphorylated product of FTY720 (Albert *et al.* 2005). SEW2871 [5-(4-phenyl-5-trifluoromethylthiophen-2-yl)-3-(3-trifluoromethylphenyl)-(1,2,4)-oxadiazole] was used as a S1P1 selective compound reported to have a nanomolar affinity for S1P1 (Sanna *et al.* 2004; Bolick *et al.* 2005; Jo *et al.* 2005; Karliner 2005). AUY954 is also a S1P1 selective compound with EC50 values of approximately 0.5 nmol/L in fluorescent imaging plate reader and GTPγS assays, using Chinese Hamster Ovary (CHO) cell lines stably expressing S1P receptors (Pan *et al.* 2006).

Mixed rat cortical primary cultures

Cortical cultures were prepared from embryonic day-18 Sprague–Dawley rats. Cortices were dissected in cold Hepes-buffered saline solution (HBSS) and then dissociated using trypsin for 10 min at 37°C. Cultures were plated onto poly-L-lysine-coated Lab-Tek II eight-well-chambered coverglasses (Nalge Nunc International; Naperville, IL, USA) and maintained at 37°C, 5% CO_2 in Neurobasal media with B27 supplement (Invitrogen AG, Basel, Switzerland). Mixed cultures were used after 7 days *in vitro*. Animal handling was conducted in accordance with Swiss animal welfare authorities.

Rat astrocyte cultures

Primary astrocyte cultures were prepared from postnatal day-2 Sprague–Dawley rats as follows: whole brains where dissected in cold HBSS (Invitrogen), 0.15% $MgSO_4$, then dissociated using trypsin for 10 min at 37°C. After trituration, cells were resuspended in Basal Medium Eagle (Invitrogen), 10% horse serum (Invitrogen) and plated onto poly-D-lysine coated flasks (Beckton Dickinson Labware) and maintained at 37°C, 5% CO_2. After 2 days, cultures were washed and the medium (Basal Medium Eagle, horse serum) replaced. Then medium was changed every 2 days until day 9, when cultures were shaken to remove oligodendrocytes, and remaining astrocytes were trypsinized and replated onto either poly-D-lysine-coated 24-well plates, or eight-well chambered coverglasses (Lab-Tek II; Nalge Nunc International).

Calcium signaling

Cells were grown on Lab-Tek II eight-well-chambered coverglasses (Nalge Nunc International). Cells were loaded with 1 μmol/L Fluo-4 AM (Invitrogen) in HBSS containing probenecid (0.5 μmol/L) at 37°C for 15 min. Cells were washed, and fresh HBSS (0.5 μmol/L probenecid) was added. Calcium responses were analyzed in a laser-scanning microscope (LSM510 META; Carl-Zeiss AG, Feldbach, Switzerland) at 20°C; scanning speed was 2 frames/s. Stimulation of cells was performed by adding a 4× concentrated solution (in HBSS, Probenecid) of S1P, FTY720P or glutamate to the cells with a manual pipette. Calcium traces were analyzed in the indicated areas of interest (arrows in mixed cultures) or averaged over the entire window (pure astrocytes) using the Zeiss LSM510 standard software.

Immunocytochemistry

Fixation and permeabilization of cells was carried out using the Cytofix/Cytoperm kit (BD Biosciences, Palo Alto, CA, USA). Chambers were washed with phosphate-buffered saline, Cytofix/Cytoperm solution was added (300 μL/well, eight-well chambered coverglasses) and the samples were fixed at 4°C for 20 min. The wells were then rinsed twice with washing solution (1×, diluted from 10× stock; BD Biosciences) and blocking buffer (140 mmol/L NaCl, 10 mmol/L HEPES, 5 mmol/L $CaCl_2$, 5% fetal calf serum, 1% bovine serum albumin, 0.04% NaN_3 in washing solution) was added (300 μL/well) for at least 20 min at 20°C. Staining of organotypic hippocampal slices is described in the corresponding section. Primary antibodies were diluted into blocking buffer and left on the samples overnight at 4°C. Monoclonal anti-glial fibrillary acidic protein (GFAP) (MAB360, dilution 1 : 500), monoclonal anti-CNPase (MAB326, dilution 1 : 200) and monoclonal anti-CD11b (CBL 1512, dilution 1 : 200) were from Chemicon International (Temecula, CA, USA), and monoclonal anti-βIII-tubulin was from R&D Systems Inc. (Minneapolis, MN, USA; MAB1195, dilution 1 : 500). Cells

were rinsed twice and incubated with anti-rabbit and/or anti-mouse secondary antibodies coupled to Alexa 488 or Alexa 633 fluorescent dyes (Invitrogen) for 2–3 h at 20°C, washed and viewed with a Zeiss LSM 510 META confocal microscope (Carl-Zeiss AG); nuclei were counter-stained with 4′,6-diamidino-2-phenylindole.

Quantitative real-time PCR

Whole rat brain (postnatal day 2) or astrocyte cultures were disrupted in RNeasy Lysis Buffer (Qiagen, AG, Hombrechtikon, Switzerland) using a TissueLyser (Qiagen). RNA purification was performed with TRIzol (Invitrogen) according to the manufacturer's protocol. Total RNA was further purified including a DNAse I treatment using the RNeasy Minelute kit (Qiagen). Five microgram of total RNA was processed to a reverse transcription using the Superscript III kit (Invitrogen) and a mix of dN6 and oligodT oligonucleotides. A minus reverse transcriptase control was kept to monitor the potential remaining genomic contamination. The real-time PCR was done using a Rotor-Gene RG-3000A (Corbett Research UK Ltd, Cambridge, UK). 1/50 000 SybrGreen (Invitrogen) was added to the PCR mix (Platinum TAQ polymerase 0.25 U, 2.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTP, 0.3 mmol/L primers, cDNA equivalent to 50 ng total RNA). After a 2 min denaturation at 95°C, the PCR cycling was 95°C for 10 s, 55°C for 15 s, and 72°C for 20 s. The primer used (5′–3′) were S1P1_sense: TGTCCTCCACCAGCATCCCA, S1P1_antisense: ATGGGCCGGTGGAACTTCT, S1P2_sense: ATGGGCGGTTTATACTCA, S1P2_antisense: AAGGTTCTCCACCACGAT, S1P3_sense: CGCCAGTCTTGGGGAATGA, S1P3_antisense: GCCAAGTTGCCGATGAAA, S1P4_sense: GAACATCAGTACCTGGTCC, S1P4_antisense: GTTTTCCAGCACCACCAG, S1P5_sense: GTGAGCGAGGTCATCGTCCT, S1P5_antisense: TGCATGGAAGCGAGGATGG, GFAP_sense: TTGCTGGAGGGCGAAGAA, GFAP_antisense: TTGTGCTC CTGCTTCGAC, 18S_sense: TTAGAGTGTTCAAAGCAGGC, 18S_antisense: CGGTCCAAGAATTTCACC. All transcripts were processed in a single run. 1 : 50 diluted cDNA was used with 18S primers. Values provided by the software as calculated by the comparative quantitation methods were normalized to 18S.

cAMP and inositol-phophates formation

Astrocytes plated in 24-well plates were grown to 70–90% confluence and used for assaying cAMP as described (Salomon 1979). Briefly, cells were loaded with 0.75 μCi of [^{3}H]-adenine/well in serum-free Minimum Essential Medium α (Invitrogen) for 4 h; where indicated, pertussis toxin (PTX; 100 ng/mL) was also added to the medium. Cells were washed with HBSS and cAMP accumulation was measured over 15 min (HBSS, 37°C) in the absence or presence of Forskolin (Fsk; 20 μmol/L, Sigma-Aldrich, Buchs, Switzerland) and receptor agonists as indicated in figure legends. To inhibit phosphodiesterase activity, isobutylmethylxanthine (1 mmol/L, Sigma-Aldrich), Rolipram (10 μmol/L, BIOMOL International, Exeter, UK) and BAY 60–7550 (1 μmol/L, Alexis Corporation, Lausen, Switzerland) were present in all samples. Extraction and measurement of [^{3}H]-cAMP was carried out as described (Salomon 1979). For determination of inositol-phophates (IP), cells were labeled with 0.75 μCi of myo[^{3}H]inositol in serum-free Minimum Essential Medium α for 15–20 h; where indicated, PTX (100 ng/mL) was also added. Cells were washed and HBSS containing lithium (20 mmol/L) was added to block inositol monophosphatase activity, leading to accumulation of IP1. The samples were incubated at 37°C for 20 min, then cells were extracted with ice-cold formic acid and total IPs separated from free inositol using batch column chromatography (Seuwen *et al.* 1988).

Scratch-wound assay and astrocyte proliferation assay

Purified cortical astrocytes were prepared from postnatal day 2–3 mouse brains. Briefly, meninges were removed from cortices and dissociated mechanically by trituration. Suspended cells were passed through a cell strainer (100 μm) and cultured for 9–10 days until confluent in Dulbecco's Modified Eagle Medium (high glucose, Invitrogen) supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were shaken at 280 rpm on a rotating platform for 6–8 h at 37°C and then treated with 10 μmol/L cytosine arabinoside for 48 h. The protocol yielded >95% pure astrocyte cultures. Astrocytes were plated onto 2-well chamber slides and cultured in serum free Dulbecco's Modified Eagle Medium for 48 h. Following serum starvation, media was removed and a reference line was drawn at the bottom of each slide. A scratch was made in the astrocyte monolayer with a 1 mL pipette tip along the reference line. Cells were washed twice with phosphate-buffered saline to remove cell debris and fresh media containing interferon-gamma and Transforming Growth Factor α (100 and 10 ng/mL) or S1P receptor agonists was added. Images were taken at 0 and 72 h with the same field recorded in each case. To determine the area of migration the leading edge of the scratched cells at 0 h was superimposed onto the image taken at 72 h (Axiovision software, Zeiss). Measurements from quadruplicates were averaged to determine the area of migration. A colorimetric assay (MTT, Chemicon International) to assess proliferation was performed under similar conditions in 96-well plates.

Hippocampal organotypic slice cultures

Hippocampal slices were cultured according to the protocol of Stoppini (Stoppini *et al.* 1991). Briefly, the hippocampi of P7 rats were dissected and cut into 350-μm-thick slices. Slices were separated in ice cold dissecting medium and placed into Millicel-CM inserts (Millipore, Billerica, MA, USA) with 1 mL of culture medium in six-well plates (TPP, Trasadingen, Switzerland). Cultures were treated with FTY720P (1 nmol/L or 5 nmol/L) or SEW2871 (100 nmol/L) for 1 week at 33°C, 5% CO_2. Slices were fixed with 4% paraformaldehyde overnight and then permeabilized overnight in permeabilization buffer [Tris-buffered saline, supplemented with 0.2% TopBlock (Juro AG, Luzern, Switzerland), 1.5% horse serum, and 0.3% Triton X-100]. Primary antibody incubation with mouse monoclonal anti-GFAP (Chemicon, MAB360, dilution 1 : 500) was performed in the permeabilization buffer for 5 days. Secondary antibody, Alexa 488 anti-mouse (Invitrogen), was incubated for 2 h. Cultures were then imaged with a Zeiss Axioskop2 mot plus. The number of GFAP positive cells present beyond the edge of the culture was counted.

Results

FTY720P elicits calcium signals in astrocytes

As S1P receptors are widely expressed in the CNS (Chun *et al.* 2000), we probed for calcium signals triggered by FTY720P

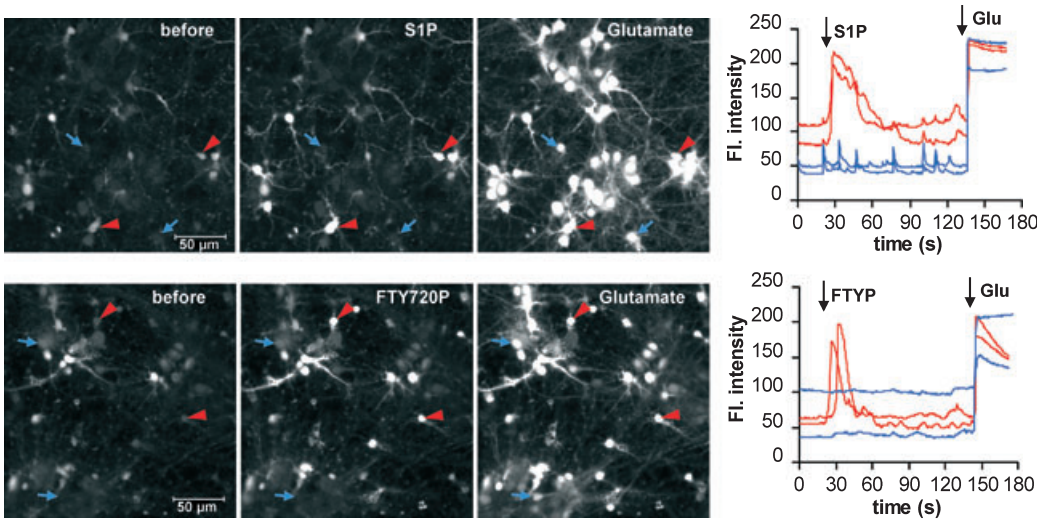

Fig. 1 S1P and FTY720P elicit a calcium response in mixed cortical cultures. Fluo-4-loaded mixed cortical cultures (rat) were stimulated with S1P (100 nmol/L, upper panels) or FTY720P (100 nmol/L, lower panels). Representative images were taken from a time–lapse series before addition (left), after addition of S1P or FTY720P (middle), and after stimulation with glutamate (1 mmol/L, right). The red arrowheads indicate representative cells responding to S1P or FTY720P, blue arrows point to cells only responding to glutamate; the graphs show the fluorescence intensity measured over time

and S1P in mixed cortical cultures from rat embryos. We found that both S1P (100 nmol/L) and FTY720P (100 nmol/L) elicited calcium signals in a sub-population of cells, while all cells responded to glutamate (1 mmol/L) (Fig. 1). To identify the cell type responding to FTY720P, we performed Ca^{2+} imaging and, consecutively, immunocytochemical analysis. We observed FTY720P-evoked Ca^{2+} signals in GFAP immunopositive astrocytes (Fig. 2a), but not in neurons stained for beta-III tubulin (Fig. 2b).

FTY720P stimulates calcium signals via S1P receptors

We next determined whether the Ca^{2+} signals evoked by FTY720P and S1P in astrocytes were mediated via a common signaling pathway in our experimental conditions. We pre-stimulated the mixed cultures with a high concentration of S1P (1 µmol/L) to saturate all S1P receptors prior to the addition of a high concentration of FTY720P (1 µmol/L). We found that pre-stimulation by S1P abrogated any further response to FTY720P, but not to glutamate (Fig. 3). These results strongly suggest that the FTY720P-elicited Ca^{2+} signals in astrocytes are mediated via S1P receptors.

Characterization and the effects of FTY720P on pure astrocyte cultures

To learn which S1P receptor subtypes are involved in FTY720P-mediated signaling in astrocytes, we first characterized the expression profile using pure astrocyte cultures. Immunocytochemical analysis showed that these cultures contained mainly astrocytes (>95%) with few oligodendrocytes, neurons, and microglia (Fig. 4a). In agreement, real-time PCR showed a 20-fold enrichment of GFAP mRNA in our astrocyte cultures when compared with whole brain tissue (*data not shown*). The pure astrocyte cultures expressed S1P receptor mRNA with a rank order of S1P1 ≈ S1P3 >> S1P2 > S1P5 and no S1P4. An enrichment of S1P3 mRNA was observed when compared with whole brain tissue (Fig. 4b). Our pure astrocytes showed S1P- and FTY720P-evoked Ca^{2+} signals (Fig. 4c) thus confirming the results obtained using the mixed cortical cultures.

FTY720P-mediated inhibition of cAMP formation in astrocytes

Having found a relatively high expression of S1P1 and S1P3 receptors in astrocytes, we next aimed to dissect the contributions of both subtypes in FTY720P-mediated signal transduction. As all known S1P receptors couple to Gi proteins, we first determined the attenuation of Forskolin-induced cAMP formation as a direct measure of receptor activation. We found that sub-nanomolar concentrations of FTY720P effectively inhibited cAMP formation (Fig. 5a). In contrast, a comparable inhibition by S1P required at least 1000-fold higher concentrations. To specifically assess the role of S1P1 receptors, we examined the activity of the S1P1-selective agonists SEW2871 (Jo *et al.* 2005) and AUY954 (Pan *et al.* 2006). Both agonists effectively attenuated Forskolin-induced cAMP formation to a comparable extent as S1P and FTY720P (Fig. 5a). The effects of all agonists were concentration-dependent and sensitive to PTX (Fig. 5b), demonstrating the involvement of Gi proteins. These data suggest that activation of S1P1 receptors is

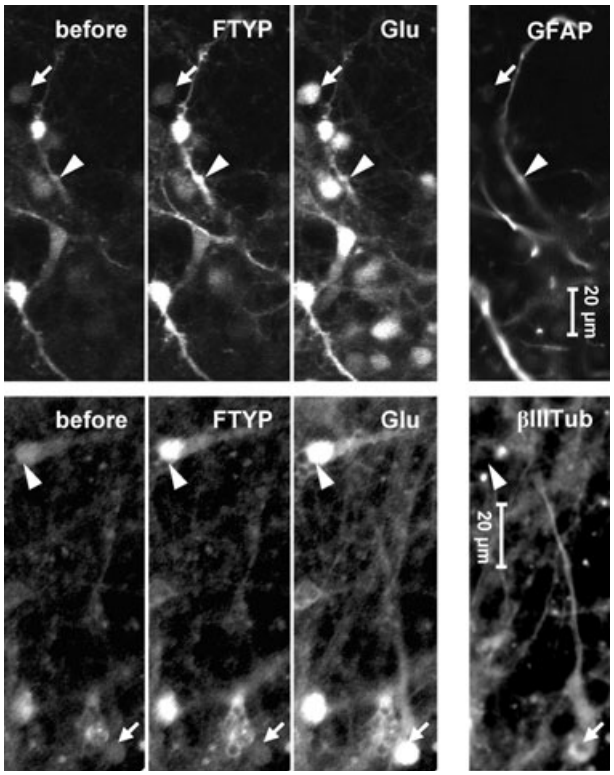

Fig. 2 Identification of astrocytes as cells responding to FTY720P in mixed cortical cultures. Images are taken from time–lapse series and show Ca^{2+} signals before stimulation and after consecutive stimulation with FTYP (FTY720P, 100 mol/L) and then Glu (glutamate, 1 mmol/L). After calcium imaging, the cells were fixed and stained against GFAP to detect astrocytes or against βIII-tubulin to label neurons (βIII-tubulin). Arrowheads indicate FTY720P-responsive cells (positive for GFAP, negative for βIII-tubulin) and arrows point to cells responding only to glutamate (negative for GFAP, positive for ßIII-tubulin). Note that FTY720P responsive cells were all positive for GFAP but never for βIII-tubulin.

sufficient to account for the effects mediated by FTY720P on cAMP formation.

Stimulation of IP formation by FTY720P in astrocytes

Sphingosine-1-phosphate 1 receptors are exclusively coupled to PTX-sensitive Gi proteins, whilst S1P3 receptors are predominantly coupled to Gq proteins (Okamoto *et al.* 1998, 1999). The activation of phospholipase C (PLC) and the formation of IP is generally mediated by the α subunits of Gq proteins, however activation can also occur via the βγ subunits of Gi proteins. We compared the effects of S1P and FTY720P on the activation of PLC in the presence and absence of PTX. We found that S1P (1 µmol/L) caused 10-fold increase in IP levels, which was in part sensitive to PTX indicating the involvement of both Gi and Gq protein-coupled receptors. In contrast, FTY720P (100 nmol/L) maximally induced a four-fold increase in IP levels, that was largely sensitive to PTX (Fig. 6). The data suggest that FTY720P activates Gi proteins with high efficiency thus allowing a stimulation of IP formation, with minor involvement of Gq proteins. The relatively high concentrations of FTY720P needed to stimulate IP formation likely results from the poor efficiency of Gi-coupled S1P1 receptors to activate PLC. Whilst the inhibition of adenylyl cyclase (AC) by active α subunits of Gi proteins occurs at low receptor occupancies, a higher number of activated receptors and high concentrations of βγ subunits are required to activate PLC (Birnbaumer 1992). The results are also consistent with a partial agonism of FTY720P on Gq-coupled S1P3 receptors, as confirmed in CHO cells stably expressing human S1P3 receptors (*see inset,* Fig. 6). Furthermore, the selective S1P1 receptor agonists SEW2871 and AUY954, which exclusively induce activation of Gi proteins, elicit a moderate two-fold increase in IP formation that is

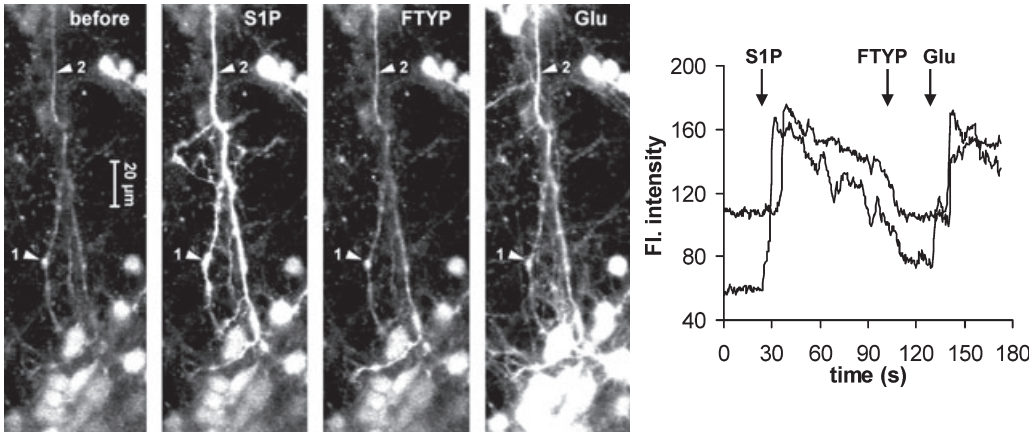

Fig. 3 Sphingosine-1-phosphate treatment desensitizes the calcium responses elicited by FTY720P. The calcium images shown were taken from a time–lapse series of mixed cortical cultures before stimulation (first panel), after addition of S1P (1 µmol/L, second panel), after subsequent addition of FTY720P (1 µmol/L, third panel) and finally after adding glutamate (1 mmol/L, fourth panel) as a positive control. Arrowheads indicate two areas of interest, the corresponding traces of the fluorescence intensities over time are shown in the graph.

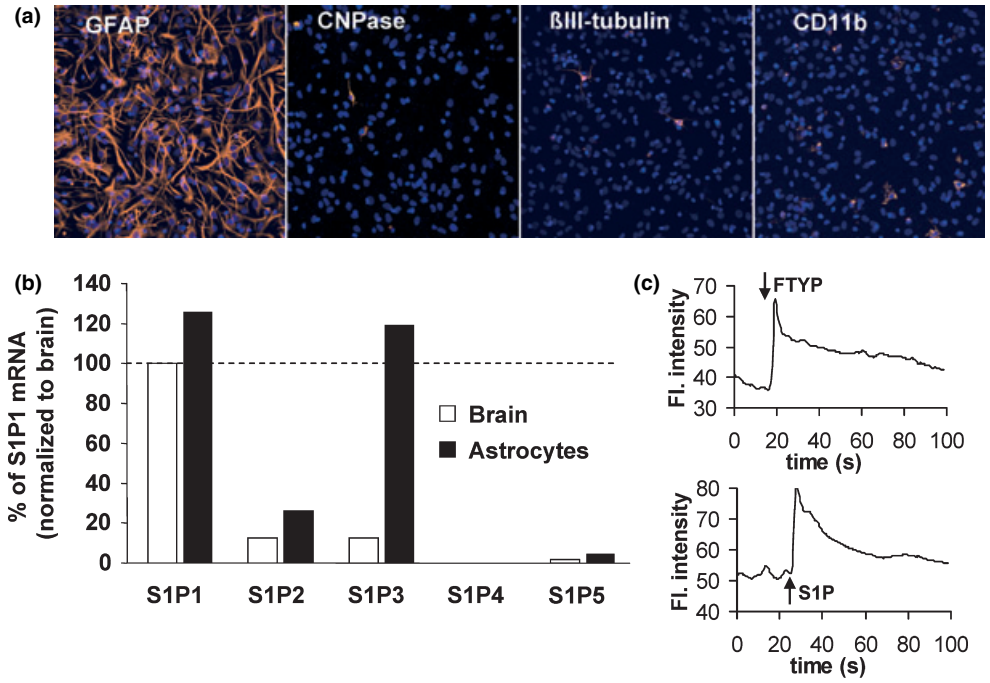

Fig. 4 Detection of S1P1 receptor and calcium responses in enriched astrocyte cultures. (a) The enriched astrocytes cultures were stained with cell type specific antibodies against GFAP (astrocytes), CNPase (oligodendrocytes), βIII-tubulin (neurons), and CD11b (microglia). Images were taken at low magnification (20× objective) to visualize a large number of cells (DAPI stained nuclei, blue). (b) The mRNA expression profile of S1P receptor subtypes in rat whole brain and pure cortical astrocyte cultures. Shown is a representative experiment. All values are presented as percentage of S1P1 mRNA found in whole brain, which was defined as 100%. (c) Recordings of the mean fluorescence intensity of a full field over time derived from calcium imaging in enriched astrocyte cultures.

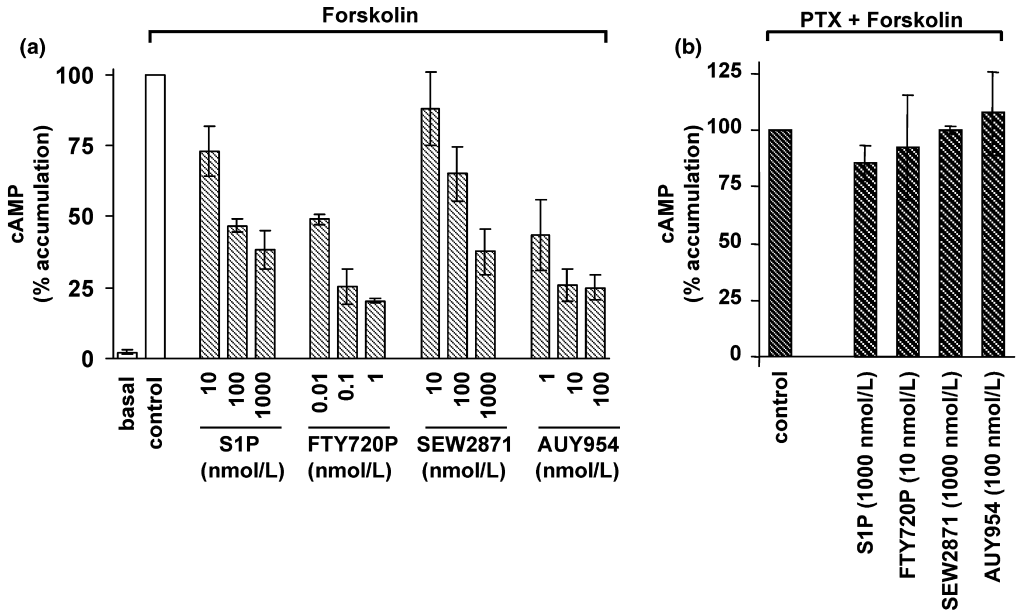

Fig. 5 FTY720P inhibits cAMP formation in pure astrocytes cultures via Gi proteins. (a) Astrocytes were stimulated with Forskolin in the absence or presence of the indicated concentrations of S1P receptor agonists. The cAMP levels are expressed as percentage of the Forskolin control and represent mean ±SEM of three independent experiments performed in triplicates. (b) Prior to stimulation with Forskolin and the indicated S1P receptor agonists, astrocytes were treated with PTX (100 ng/mL, 4 h). The cAMP levels are expressed as percentage of the Forskolin control and represent mean ±SEM of at least two independent experiments performed in triplicates.

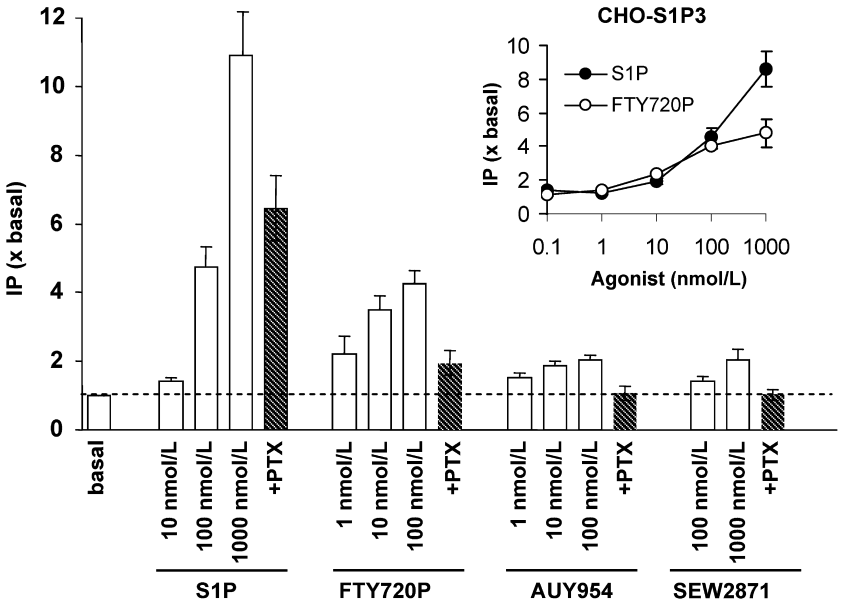

Fig. 6 FTY720 stimulates IP formation in astrocytes. (a) Astrocytes were stimulated with the indicated S1P receptor agonists and IP levels were determined after 20 min. Where indicated, astrocytes were treated with PTX (100 ng/mL, overnight) prior to stimulation (striped bars). IP levels are expressed as a multiple of non-treated control (baseline, indicated by broken line) and represent mean ±SEM of three independent experiments performed in triplicates. *Inset* shows the concentration–response relationships for S1P and FTY720P-induced IP formation in CHO cells stably expressing the S1P3 receptor. Shown is the mean ±SEM of three independent experiments.

completely abolished after PTX treatment. The higher IP levels elicited by FTY720P can be explained by the synergism of Gi-mediated IP formation and the partial activation of Gq-coupled S1P3 receptors. A synergistic effect of Gi- and Gq proteins on PLC activity has been previously described in various cells including primary astrocytes (Biber *et al.* 1997, Dickenson and Hill 1998). Taken together, we conclude that the FTY720P-induced activation of PLC occurs mainly via S1P1/Gi which is enhanced by a weak but synergistic component of S1P3/Gq signaling.

FTY720P promotes astrocyte migration

Finally, we studied the effects of FTY720P on astrocyte migration. Previous reports have suggested that S1P1 and S1P3 receptor activation promotes cell migration in transfected CHO cells and endothelial cells. In contrast, S1P2 receptors play an inhibitory role in migration of heterologous cells and smooth muscle cells (Kon *et al.* 1999; Kimura *et al.* 2000; Okamoto *et al.* 2000; Lee *et al.* 2001; Tamama *et al.* 2001). We found that FTY720P increased cell migration in a scratch-wound assay using a monolayer of pure astrocytes (Fig. 7a). Both FTY720P (10 nmol/L) and the S1P1 selective agonist SEW2871 (100 nmol/L), promoted astrocyte migration, to a similar extent as the positive control treated with interferon-gamma and Transforming Growth Factor α (100 and 10 ng/mL). We observed no effects of FTY720P or SEW2871 on cell numbers by flourimetric viability assay (Fig. 7a). To support this data, hippocampal organotypic slice cultures were treated with FTY720P and astrocyte migration was assessed by GFAP immunostaining. We noted an increase in the number of astrocytes migrating beyond the slice edge when treated with FTY720P (1 or 5 nmol/L) (Fig. 7b). These data show that FTY720 and the activation of S1P1 receptors promote astrocyte migration in primary cultures.

Discussion

Fingolimod (FTY720) has recently entered phase III clinical trials for the treatment of MS. The drug is phosphorylated *in vivo* to yield the active metabolite, FTY720P, which is a potent agonist on all S1P receptors except S1P2 (Brinkmann *et al.* 2002). The endogenous ligand, S1P, is known to evoke signaling events in astrocytes, including the inhibition of AC, stimulation of PLC, elevation of intracellular Ca^{2+}, and an increase in ERK phosphorylation (Pebay *et al.* 2001; Malchinkhuu *et al.* 2003; Rao *et al.* 2003, 2004; Sorensen *et al.* 2003). In this report, we show that FTY720P triggers a number of signaling events in astrocytes predominantly via S1P1 receptors whereas S1P additionally involves S1P3 receptors. Importantly, we demonstrate a functional role of FTY720P in astrocyte migration which is mimicked by a S1P1 selective agonist.

Using mixed cortical cultures we find an FTY720P-evoked intracellular Ca^{2+} elevation in astrocytes but not in

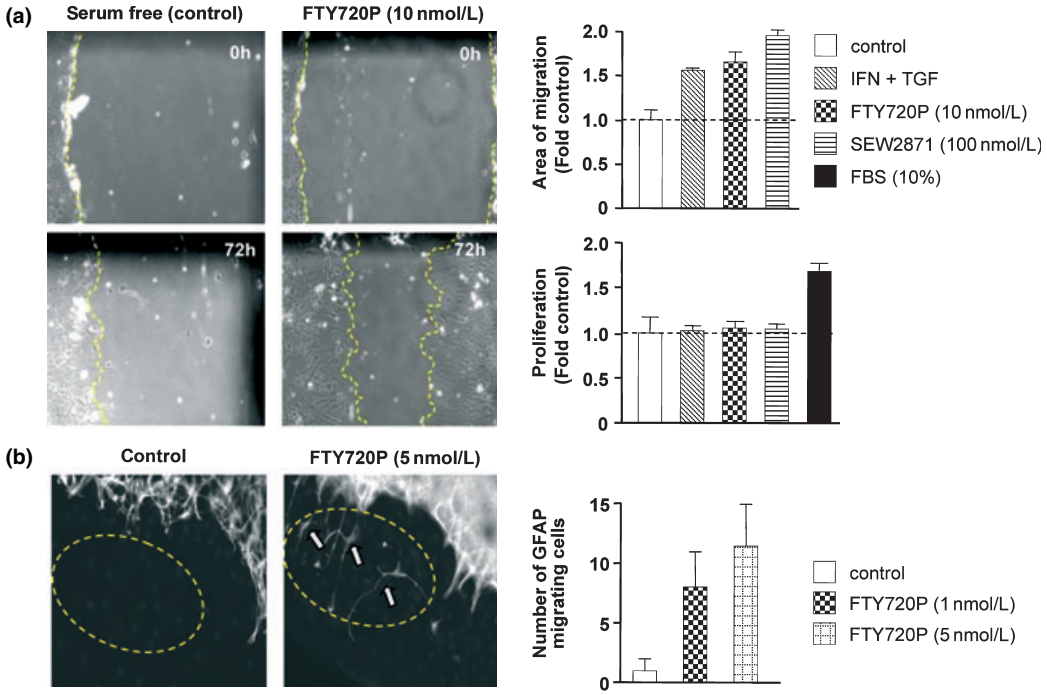

Fig. 7 FTY720 stimulates astrocyte migration. (a) Scratch-wound assay. Astrocytes were stimulated with cytokines (IFNγ and TGFα) or the S1P receptor agonists at indicated concentrations for 72 h following serum starvation. Average area of astrocyte migration was measured and compared with the serum free condition. The depicted area of migration is representative of three independent experiments performed in quadruplicates (*upper graph*). Astrocytes seeded onto a 96-well plate, were serum starved for 48 h and subsequently treated with indicated cytokines or S1P receptor agonists. Cell numbers were measured using a colorimetric (MTT, Chemicon) assay for cell survival and proliferation and compared with proliferation observed with 10% FBS. Results are average of three independent experiments (*lower graph*). (b) Hippocampal organotypic slice cultures were incubated with FTY720P (1 or 5 nmol/L) for 1 week. After fixation and GFAP immunohistochemistry, the number of cells labeled beyond the edge of the cultures was counted. The number of migrating cells is presented as an average of three independent experiments.

neurons. Stimulation with a high concentration of S1P prevents FTY720P from eliciting any further Ca^{2+} response, most likely by the saturation of common S1P receptors as demonstrated by the strong intracellular Ca^{2+} elevation triggered by a subsequent application of glutamate. We did not detect any FTY720P or glutamate-evoked Ca^{2+} signals in oligodendrocytes which were, however, present at low numbers in the mixed cell preparations (not shown). The possible effects of FTY720P-mediated signaling in oligodendrocytes and microglia are therefore likely to be better studied in culture systems enriched for these cell types.

Expression profiling of S1P receptors in enriched astrocyte cultures show high levels of S1P1 and S1P3, with little or no mRNA for S1P2, S1P4, and S1P5, in agreement with previous observations (Pebay *et al.* 2001; Malchinkhuu *et al.* 2003; Rao *et al.* 2003, 2004; Sorensen *et al.* 2003; Anelli *et al.* 2005; Bassi *et al.* 2006). The expression profile and the pharmacological properties of FTY720P imply a key role for S1P1 receptors in mediating the response to FTY720P in astrocytes. The results also suggest that astrocytes are directly stimulated by FTY720P rather than by chemical communication and/or physical interaction with neurons and oligodendrocytes.

The S1P1 receptor exclusively couples to Gi proteins while the S1P3 receptor couples to both Gq and Gi proteins (Okamoto *et al.* 1999; Windh *et al.* 1999). We show FTY720P inhibits cAMP formation in a PTX sensitive manner, demonstrating the involvement of Gi protein-coupled receptors. FTY720P effectively inhibits cAMP formation at 1000-fold lower concentrations compared with S1P, which may be attributed to better a coupling efficiency of FTY720P-bound S1P1 receptors and/or a rapid turnover of S1P by astrocytes. As FTY720P is only a partial agonist on S1P3 receptors (Brinkmann *et al.* 2002), we suggest that the inhibition of AC by FTY720P occurs mainly via the S1P1 receptor. In support of this notion, we find that both S1P1-selective agonists, SEW2871 and AUY954, are as efficacious as FTY720P in attenuating cAMP formation.

To further assess the signaling of FTY720P via S1P3 receptors on astrocytes, we measured the formation of IP

which is catalyzed by PLC. Ca^{2+} release downstream of G protein-coupled receptors typically occurs by Gαq-mediated activation of PLC-β1-4, leading to the formation of IP3 that in turn triggers liberation of Ca^{2+} from intracellular stores. The activation of Gi proteins can also elicit a Ca^{2+} response via βγ subunit-mediated stimulation of PLC-β2,3 (Birnbaumer 1992; Katz *et al.* 1992; Exton 1997). We treated astrocytes with PTX to dissect the role of S1P1/Gi and S1P3/Gq in FTY720P-induced IP formation. In these experiments FTY720P partially elevated IP formation, that was largely sensitive to PTX, in contrast to S1P. The data clearly indicate that a small portion of IP formation is mediated by S1P3/Gq signaling, which is consistent with partial agonism of FTY720P on S1P3 receptors. Furthermore, the IP formation induced by S1P1 selective agonists was fully attenuated by PTX treatment. Taken together, our data show that FTY720P activates S1P1/Gi signaling to AC and PLC in primary astrocytes and extends previous studies on S1P-evoked signal transduction in heterologous cells (Okamoto *et al.* 1998, 1999).

Finally, FTY720P stimulates the migration of astrocytes in both dissociated cell monolayers and brain slices. As FTY720P is applied to the incubation media, the migratory responses likely reflect an increased chemokinetic mobility of cells rather than directional movement along a concentration gradient. The fact that a S1P1 selective agonist, SEW2871, induces similar responses indicates a key role for S1P1 receptors. These data are in agreement with previous reports showing that S1P1 receptor activation promotes cell migration in various cell types, including endothelial cells, embryonic fibroblasts, and heterologous cells (Kon *et al.* 1999; Liu *et al.* 2000; Okamoto *et al.* 2000; Lee *et al.* 2001; Tamama *et al.* 2001). Moreover, S1P has been shown to evoke a chemotactic response in primary Schwann cells and to increase chemokinetic mobility (Barber *et al.* 2004).

There are two important differences between S1P and FTY720P. Firstly, S1P is a full agonist on S1P2 and S1P3 receptors whereas FTY720P does not bind S1P2 and is a partial agonist on S1P3 receptors. As S1P2 receptors inhibit migration (Okamoto *et al.* 2000) the effects of S1P likely result from a balance of S1P1 and S1P2 activity. In contrast, FTY720P can be assumed to promote migration without exerting a S1P2-associated inhibitory component. Secondly, the FTY720P-bound S1P1 receptors exhibit a better coupling efficiency to Gi proteins, compared with S1P-bound receptors. Therefore, we suggest that FTY720 strongly stimulates Gi signaling via S1P1 (and not S1P3) which promotes astrocyte migration. In support of this notion, studies using embryonic fibroblasts from S1P1 knock out mice are refractory to S1P-induced migration, while still expressing normal levels of S1P2 and S1P3 receptors (Liu *et al.* 2000).

Other functional effects of S1P and its receptors on astrocytes have been reported. For example, astrocytes have the ability to release S1P in response to fibroblast growth factor and tumor necrosis factor α (Riboni *et al.* 2000; Vann *et al.* 2002; Anelli *et al.* 2005; Bassi *et al.* 2006) and in turn S1P stimulates the proliferation of astrocytes in an ERK dependent mechanism (Pebay *et al.* 2001; Malchinkhuu *et al.* 2003; Sorensen *et al.* 2003; Yamagata *et al.* 2003). In our scratch wound migration assay, we did not observe an increase in astrocyte cell numbers when treated with FTY720P, possibly due to its lack or partial activity on S1P2 and S1P3 receptors, respectively. *In vivo*, the injection of S1P into the striatum of mice results in an increase of GFAP staining (Sorensen *et al.* 2003) and the exposure of astrocytes to S1P results in the release of growth factors such as glial cell-line derived neurotrophic factor, ciliary neurotrophic factor and fibroblast growth factor (Sato *et al.* 1999, 2000; Malchinkhuu *et al.* 2003; Yamagata *et al.* 2003; Bassi *et al.* 2006). Similar brain injection studies remain to be performed in order to compare the effects of FTY720P with S1P *in vivo*.

In conclusion, our results show that FTY720P triggers many of the intracellular signaling pathways akin to S1P in cultured astrocytes. Further characterization of the subtle pharmacological and functional differences between S1P and FTY720P may be important in understanding the possible involvement of astrocytes in the beneficial effects of FTY720 in patients suffering from MS.

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