Down-regulation of S1P1 Receptor Surface Expression by Protein Kinase C Inhibition*

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The sphingosine 1-phosphate receptor type 1 (S1P1) is important for the maintenance of lymphocyte circulation. S1P1 receptor surface expression on lymphocytes is critical for their egress from thymus and lymph nodes. Premature activation-induced internalization of the S1P1 receptor in lymphoid organs, mediated either by pharmacological agonists or by inhibition of the S1P degrading enzyme S1P-lyase, blocks lymphocyte egress and induces lymphopenia in blood and lymph. Regulation of S1P1 receptor surface expression is therefore a promising way to control adaptive immunity. Hence, we analyzed potential cellular targets for their ability to alter S1P1 receptor surface expression without stimulation. The initial observation that preincubation of mouse splenocytes with its natural analog sphingosine was sufficient to block Transwell™ chemotaxis to S1P directed subsequent investigations to the underlying mechanism. Sphingosine is known to inhibit protein kinase C (PKC), and PKC inhibition with nanomolar concentrations of staurosporine, calphostin C, and GF109203X down-regulated surface expression of S1P1, but not S1P4 in transfected rat hepatoma HTC4 cells. The PKC activator phorbol 12-myristate 13-acetate partially rescued FTY720-induced down-regulation of the S1P1 receptor, linking PKC activation with S1P1 receptor surface expression. FTY720, but not FTY720 phosphate, efficiently inhibited PKC. Cell-based efficacy was obvious with 10 nM FTY720, and in vivo treatment of mice with 0.3–3 mg/kg/day FTY720 showed increasing concentration-dependent effectiveness. PKC inhibition therefore may contribute to lymphopenia by down-regulating S1P1 receptor cell surface expression independently from its activation.

Lymphocyte circulation is dependent on cell surface expression of the S1P1 receptor (1). Its endogenous ligand S1P serves as an exit signal in blood and lymph for lymphocytes that are leaving lymph nodes and thymus (2). The lymphocytes are thought to migrate from lymphoid organs with no or low S1P levels to blood and lymph with high S1P levels along a proposed S1P gradient (3). Deletion or down-regulation of the S1P1 receptor in lymphocytes prevents their exit from thymus and lymph nodes and renders mice lymphopenic in blood and lymph (1, 4). Premature internalization of the S1P1 receptor is induced by pharmacological agonists (5–7) and by inhibition or deletion of the S1P-degrading enzyme S1P-lyase (8, 9). In both cases the concentration of the endogenous or synthetic S1P1 receptor agonist is predominantly increased in lymphoid organs and prevents re-expression of S1P1 on the surface of lymphocytes (10). The exit signal S1P is consequently not recognized anymore, and lymphocytes are trapped in thymus and lymph nodes (11). Furthermore endothelial cells are stimulated via S1P, and close postulated endothelial cell barriers, again preventing lymphocytes from passing (12–14). Regulation of S1P1 receptor surface expression is therefore crucial for maintaining lymphocyte circulation and immune surveillance.

The immunomodulator Fingolimod (2-amino-2-[(4-octylphenyl)ethyl]-1,3-propanediol, FTY720) is a prodrug in phase III clinical trials for treatment of multiple sclerosis that serves as an agonist for S1P1 after phosphorylation (6, 15–17). Premature activation-induced down-regulation of S1P1 receptor surface expression on lymphocytes is thought to be the main principle for its biological activity in lymphoid organs, emphasizing the importance of regulation of S1P1 receptor cell surface expression for adaptive immune functions (1, 7, 18). It was also tested in phase III clinical trials as immunosuppressant after renal transplantsations but failed because of side effects such as bradycardia (19, 20). FTY720 phosphate (FTY-P) not only activates S1P1, but also three of the four remaining S1P receptors except S1P4 (6, 17). Furthermore FTY720 is cell-permeable and targets many other intracellular enzymes related to sphingolipid metabolism like sphingosine kinases 1 and 2 (SK1/2) (21), phospholipase A2 (PLA2) (22), S1P-lyase (23), and ceramide synthases (24). Because we originally observed a different specificity for FTY720-induced inhibition of S1P receptors compared with FTY-P induced stimulation (7), we aimed to study potential cellular targets of FTY720 involved in S1P1 receptor surface expression that are modulated independently from its phosphorylation to FTY-P.

A prominent candidate in this regard is protein kinase C (PKC), because it is inhibited by the natural analog sphingosine (Sph) (25, 26). Twelve PKC isoforms are known and are classified as conventional, novel, and atypical PKC (27). S1P1 receptor phosphorylation and subsequent internalization can be performed by PKC after activation (28), and late recovery of S1P1 surface expression on lymphocytes after long term exposure with its endogenous ligand S1P is dependent on PKCε expression (29). We therefore investigated the role of PKC for S1P1...
receptor surface expression and the influence of FTY720 on PKC activity in vitro using N-terminal hemagglutinin (HA) epitope-tagged human S1P1 (S1P1-HA) expressing rat hepatoma HTC4 cells (7), and in vivo with wild type and SK2-deficient mice that are defective for FTY720 phosphorylation (30, 31).

EXPERIMENTAL PROCEDURES

Chemicals and Mice—S1P and the PKC activator phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich, and the PKC inhibitor myr-ΨPKC (myristoylated peptide: Myr-RFARKGALRQKNV) (32) was from Promega. The PKC inhibitors calphostin C, GF109203X, and staurosporine were purchased from Biomol GmbH (Hamburg, Germany), 1 mM stock solutions were prepared in Me2SO, and aliquots were stored at −80 °C. Sph was purchased from Otto Nordwald GmbH (Hamburg, Germany). Chemicals and solvents were purchased from Roth (Karlsruhe, Germany) if not stated otherwise. AAL(R/S), FTY720, and FTY-P were kindly provided by Volker Brinkmann (Novartis, Basel, Switzerland) (17). FTY-P was dissolved at a concentration of 50 mM in Me2SO, 50 mM HCl and diluted with methanol to 1 mM FTY-P. FTY720, AAL(R/S), and S1P were dissolved in methanol to the final concentration of 1 mM. C57BL/6 mice were purchased from the Hannover Medical School Animal Facility (Hannover, Germany). SK2-deficient mice were kindly provided by Andreas Billich (Novartis). The anti-S1P antibody Sphingomab™ was liberally made available by Roger Sabbadini (LPath Inc., San Diego, CA).

Cell Culture—Serum, cell culture media, antibiotics, and supplements were purchased from PAA Laboratories (Coelbe, Germany) if not otherwise stated. Wild type rat hepatoma HTC4 cells were cultured in Eagle’s minimum essential medium with Earle’s salts supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 2X nonessential amino acids, 100 units/ml penicillin G, 100 µg/ml streptomycin, and 2 mM l-glutamine. HTC4 cells expressing human S1P1-HA or S1P1-RA were cultured in the presence of G418 sulfate (0.4 mg/ml). Primary splenocytes were incubated in RPMI 1640 supplemented with 10% fetal calf serum, 100 units/ml penicillin G, 100 µg/ml streptomycin, and 2 mM l-glutamine.

Flow Cytometry—Cell staining and analysis were performed according to standard protocols. Surface expression of S1P1-HA on HTC4 cells was analyzed by fluorescence-activated cell sorting using the FACSDiCalibur (Becton Dickinson, Heidelberg, Germany). The N-terminal HA epitope (peptide sequence: MGYPYDVDPYAGGP) was detected with the rat anti-HA antibody 3F10, kindly provided by Elisabeth Kremmer (GSF, Munich, Germany), and a goat anti-rat secondary antibody conjugated with cyanine 2 (Chemicon International, Inc.). Cell staining was performed with 12.5 µg/ml rat anti-HA and 5 µg/ml goat anti-rat-cyanine 2 in phosphate-buffered saline (PBS) at 4 °C for 60 min. Blood was isolated from wild type or SK2-deficient mice by cardiac puncture, and lymphocytes were counted with Flow Check® flow cytometry particles APC Maxi-Brite (Polysciences Europe GmbH, Eppelheim, Germany) after erythrocyte lysis and labeling with anti-CD3ε-fluorescein and anti-CD45R-PE (ImmunoTools, Friesoythe, Germany).

PepTag® Fluorescent Protein Kinase C Assay—PKC activity was tested using the PepTag® fluorescent protein kinase assay (Promega) according to the manufacturer’s protocol. This assay utilizes a brightly colored fluorescent peptide substrate that is highly specific for PKC. Phosphorylation by PKC changes the net charge of the substrate from +1 to −1, thereby allowing the phosphorylated and nonphosphorylated derivatives of the substrate to be separated by agarose gel electrophoresis. The phosphorylated species migrates toward the positive electrode, whereas the nonphosphorylated substrate migrates toward the negative electrode. The gel band containing the phosphorylated peptide can subsequently be visualized under UV light. All PepTag® PKC assay reaction components were combined on ice, and PKC activity was assayed in a final volume of 25 µl of the following mixture: 5 µl of 5X PKC reaction buffer (100 mM HEPES, pH 7.4, 6.5 mM CaCl2, 5 mM dithiothreitol, 50 mM MgCl2, 5 mM ATP), 5 µl of PepTag® C1 peptide (PLSRLTSVAAK, 0.4 µg/µl in water), 5 µl of freshly sonicated PKC activator solution (1 mg/ml phosphorylserine in water), 1 µl of peptide protection solution, 3.75 µl of water, 1.25 µl of vehicle (methanol/Me2SO) or compounds, and 4 µl of supplied PKC (2.5 µg/ml in PKC dilution buffer containing 100 µg/ml bovine serum albumin and 0.05% Triton X-100®, primarily α, β, and γ isotypes with lesser amounts of δ and ζ isotypes, purified from rat brain (33)). Before adding PKC, the mixture was preincubated at 30 °C for 2 min. After the addition of PKC, the entire reaction mixture was incubated at 30 °C for 30 min. The reaction was stopped by incubation at 95 °C for 10 min. Before loading samples on an agarose gel (0.8% agarose in 50 mM Tris-HCl buffer, pH 8.0), 2 µl of 80% glycerol was added to the sample. Electrophoresis was run at 100 V for 30 min in 50 mM Tris-HCl buffer, pH 8.0, and was imaged immediately under UV light with GeneSnap® V6.03 using the Gene Genius transilluminator (Syngene, Cambridge, UK) to avoid diffusion of PepTag® peptide. Densitometric quantification of the assay was performed with GeneTools® V3.05 (Syngene).

Preparation of Tissue or Cell Samples for PKC Assay—5 × 106 HTC4 cells were incubated for 15 h with 10–1000 nM AAL(R), AAL(S), or FTY720. HTC4 cells were detached from culture flasks with trypsin, pelleted by centrifugation at 400 g for 5 min at 4 °C, washed once with PBS, and centrifuged again as above. The pellet was suspended in 250 µl of ice-cold PBS, including a protease inhibitor mixture (Roche Applied Science) and homogenized on ice with a precooled Dounce homogenizer by 50 strokes. The lysate was centrifuged for 1 min at 4 °C and 10,000 × g in a microcentrifuge, and the supernatant was collected for PKC activity screening. For in vivo PKC activity studies, the mice were treated orally with 20 mg/kg/day DOP and 3 mg/kg/day AAL(R) or FTY720. After isolation of lymph nodes, spleen, and thymus, the organs were homogenized as described above. The crude extracts were freshly prepared for each experiment and assayed the same day they were prepared to obtain maximal activity. A total volume of 14 µl of extract was applied in each PepTag® PKC assay reaction.

Protein Kinase C Isotype Activity ELISA—The influence of FTY720 and AAL(S) on PKC activity of PKC isotypes α, βI, βII,
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γ, δ, ε, ζ, η, ι, and θ (Calbiochem/Merck KGaA, Darmstadt, Germany) was analyzed with the PKC kinase activity assay kit (Assay Designs, Ann Arbor, MI) according to the manufacturer’s protocol. Briefly, 20 units of each PKC isotype were mixed with FTY720 or AAL(S) (final concentration, 10 μM) in kinase assay dilution buffer and adjusted to 30 μl of total volume. After the addition of 10 μl of ATP solution, the mixture was transferred to a pre-equilibrated 96-well ELISA plate coated with PKC substrate, which was incubated for 90 min at 30 °C with gentle shaking every 20 min. After subsequent incubations of phospho-specific substrate antibody, anti-rabbit IgG-horseradish peroxidase conjugate, and tetramethylbenzidine substrate with intermittent washing, the absorbance at 450 nm was measured with the microplate reader Tecan Infinite® 200 (Crailshell, Germany). The results are expressed as relative PKC activity in concentrations normalized to vehicle control reactions of each PKC isotype, which were set to 100%.

FTY720 PKC Substrate Test—Phosphorylation of FTY720 by PKC was analyzed by incubation of 1.15 μl of 30 μM FTY720 with a mixture of PKC isotypes α, β1, βII, γ, δ, ε, ζ, η, ι, and θ (20 units each) for 15 h at 30 °C. The total assay volume was 25 μl, and buffer conditions were similar as described under PepTag® fluorescent protein kinase C assay procedure. FTY720 and FTY-P concentrations were analyzed by LC/MS/MS as described previously (10).

Splenocyte Isolation—Spleens from mice were minced on ice and pushed through a 70-μm mesh. Red blood cells were lysed in 138 mM NH₄Cl, 1 mM KHCO₃, and 0.1 mM EDTA for 5 min, and the remaining splenocytes were washed three times with PBS supplemented with 10% fetal calf serum. Some splenocyte suspensions were stimulated by incubation for 15 h in 10-cm cell culture dishes on 30 μl of adherent anti-CD3 and anti-CD28 monoclonal antibodies (BD Biosciences, Heidelberg, Germany). For selected experiments, the nuclei were pelleted by centrifugation at 300 × g for 4 °C for 5 min after homogenization with a Dounce homogenizer by 20 strokes on ice and washed twice with ice-cold PBS.

Transwell™ in Vitro Chemotaxis Assay—Migration of primary mouse splenocytes was analyzed in 24-well Transwell™ chambers (Costar, Cambridge, MA) with 6.5-mm diameter and 5-μm pore polycarbonate filters, which were coated on the lower side for 15 h at 4 °C with 600 μl of a 100 μg/ml solution of human collagen type IV (Sigma-Aldrich) in 0.5 M acetic acid, washed three times with 600 μl of PBS, and air-dried. The splenocytes were prepared as described above, and 2 × 10⁶ cells in 100 μl of RPMI 1640 supplemented with 0.1% fatty acid-free bovine serum albumin (U.S. Biological, Swampscott, MA), 100 units/ml penicillin G, 100 μg/ml streptomycin, 2 mM L-glutamine, and 25 mM HEPES buffer were placed on the top of the Transwell™ inserts. 600 μl of medium supplemented with 20 nM S1P or 38 nM (300 ng/ml) mouse recombinant SDF1α (CXCL-12) (Immun retina) as the chemotactic stimulus were added to the lower chamber. Migration was performed for 4 h at 37 °C in a humidified 5.0% CO₂ atmosphere incubator. The inserts were removed, and the number of migrated cells was assessed by flow cytometry using Flow Check® flow cytometry particles APC Maxi-Brite (Polysciences Europe GmbH) as an internal standard. To establish the number of cells that migrated nonspecifically, migration assays were performed in parallel in the absence of chemoattractants. The results are expressed as fold increases of specific migration over unspecific migration without chemoattractant.

Immunoprecipitation—The tissues were homogenized in 250 μl of PBS on ice with a precooled Dounce homogenizer by 50 strokes. The cells and tissue homogenates were lysed in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, pH 8.0, 1% Triton X-100, 20 mM NaF, 0.1 mM Na₃VO₄, and complete protease inhibitor mixture (Roche Applied Science) for 120 min at 4 °C. The concentration of protein was quantified using the BCA method (Pierce). For immunoprecipitation 10–50 μg of lysates were incubated with 3 μg of mouse anti-human-S1P₁ (clone H-60; Santa Cruz Biotechnology, Heidelberg, Germany) or rat anti-HA (clone 3F10) antibodies for 4 h. Subsequently, the antibody-protein conjugates were bound to a 1:1 mixture of protein A- and G-agarose beads (GE Healthcare) overnight at 4 °C. The beads were washed twice, dissolved in 50 μl of sample buffer (Invitrogen), and taken for Western blot analysis of bound proteins.

Protein Detection by Western Blot—Western blots were performed according to standard protocols. The tissues were homogenized in 250 μl of PBS on ice with a precooled Dounce homogenizer by 50 strokes. The cells and tissue homogenates were lysed in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, pH 8.0, 1% Triton X-100, 20 mM NaF, 0.1 mM Na₃VO₄, and complete protease inhibitor mixture (Roche Applied Science). The lysates (30–50 μg) were subjected to NuPage 4–12% Bis-Tris gels (Invitrogen) according to the manufacturer’s protocol, and the proteins were transferred to Hybond-P polyvinylidene difluoride membranes (GE Healthcare) by wet blotting. The membranes were subsequently blocked with 5% SlimFast chocolate powder (Allpharm-Vertriebs-GmbH, Messel, Germany) in Tris-buffered saline and probed with mouse anti-phosphothreonine (clone H-2; Santa Cruz Biotechnology), rabbit anti-human-S1P₁ (clone H-60, 2 μg/ml), rat anti-HA (clone 3F10, 2 μg/ml), or rabbit anti-human histone deacetylase 1 (clone H-51, 2 μg/ml; Santa Cruz Biotechnology) antibodies overnight. After incubation with specific horseradish peroxidase-labeled secondary antibodies against mouse (400 ng/ml; Abnova, Heidelberg, Germany), rabbit (160 ng/ml; Santa Cruz Biotechnology), and rat (160 ng/ml; Santa Cruz Biotechnology), the signals were visualized with the ECL detection system according to the manufacturer’s instructions (GE Healthcare).

Confocal Microscopy—HTCçı cells were seeded on glass coverslips in 6-well plates. The next day the cells were fixed with 5% paraformaldehyde in PBS for 15 min and permeabilized with 0.5% Triton X-100 for 5 min on ice. After two washes, the cells were stained with fluorescein isothiocyanate-labeled anti-HA Fab fragments (500 ng/ml, clone 3F10; Roche Applied Science) and 4’,6-diamidino-2’-phenylindole dihydrochloride (1 μg/ml; Roche Applied Science) for 2 h on ice. Subsequently the cells were washed an mounted on microscope slides with Mowiol 4–88. Confocal microscopic analysis was performed using a DM IRB with a TCS SP2 AOBS scan head (Leica Microsystems, Wetzlar, Germany).
**RESULTS**

Sph Blocks Transwell™ Chemotaxis to S1P—We have recently shown that Sph efficiently incorporates and accumulates in primary mouse splenocytes (35). To test whether or not Sph accumulation affects lymphocyte migration, freshly isolated primary mouse splenocytes were preincubated for 15 min with 300 nM and 1 μM Sph and, after several washes, subjected to Transwell™ chemotaxis assays. In contrast to control splenocytes, preincubation with Sph greatly inhibited chemotaxis to S1P, but not to the chemokine SDF1α (CXCL-12), the ligand for the chemokine receptor CXCR4 (Fig. 1). Similar Sph incubations with splenocytes did not result in detectable phosphorylation to S1P (35). To exclude the possibility that small amounts of S1P formed by the cells were responsible for desensitization of S1P receptors and the observed inhibition of splenocyte migration to S1P, equimolar concentrations of the anti-S1P antibody Sphingomab™ were added to the cells during preincubation with Sph. 100 nM Sphingomab™ was additionally added to the cells in the Transwell™ chamber during chemotaxis to prevent autocrine stimulation of S1P receptors by secreted S1P. Sphingomab™ was previously shown to bind and inactivate S1P (36). Indeed, 40 nM Sphingomab™ added to 20 nM S1P as stimulus completely blocked splenocyte migration in Transwell™ migration experiments (Fig. 1). Despite this S1P blocking activity of Sphingomab™, its presence did not prevent Sph-induced inhibition of splenocyte migration. These experiments therefore suggested that Sph may influence splenocyte migration to S1P in its nonphosphorylated state.

PKC Inhibitors Down-regulate the S1P1 Receptor—A critical requirement for splenocyte migration to S1P is surface expression of the S1P1 receptor. The latter is modulated by PKC among others, and PKC was shown to be inhibited by Sph (37). To test the relevance of PKC activation for S1P1 receptor surface expression, rat hepatoma HTC4 cells expressing the human N-terminal hemagglutinin epitope-tagged S1P1 receptor (S1P1-HA) were treated with the specific PKC inhibitor myr-ΨPKC. This PKC pseudosubstrate nonapeptide is a selective PKC inhibitor without cross-reactivity for other protein kinases (32). S1P1-HA surface expression was determined by flow cytometry. Compared with vehicle-treated S1P1-HA HTC4 cells, treatment with 50 nM of the PKC inhibitor myr-ΨPKC resulted in down-regulation of receptor surface expression (Fig. 2A). The effect was weaker compared with treatment with 300 nM FTY720 but clearly distinguishable from vehicle-treated control cells. Down-regulation of receptor surface expression through PKC inhibition was specific for S1P1 and not seen in the same cell system with a comparable construct bearing the S1P4 receptor (Fig. 2A). The fluorescence signal was specific for receptor surface expression and did not shift in nonexpressing control cells upon treatment with the PKC inhibitor myr-ΨPKC (Fig. 2A). To further demonstrate the influence of PKC inhibition on S1P1 receptor down-regulation, surface expression of S1P1 was analyzed after treatment with nanomolar concentrations of the additional PKC inhibitors staurosporine, calphostin C, and GF109203X. All of the PKC inhibitors induced severe down-regulation of S1P1 receptor surface expression, with 100 nM calphostin C being even more efficient than 300 nM FTY720, 50 nM staurosporine,
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**FIGURE 3.** FTY720-induced S1P₁ receptor down-regulation is partially rescued by PMA. S1P₁-HA expressing HTC4 cells were incubated for 15 h at 37 °C with 10 μM PKC activator PMA (blue line), 10 nM FTY720 (red line), or PMA combined with FTY720 (purple line). A black line represents vehicle-treated control cells, and HTC4 wild type cells are shown as negative control (green line). Similar incubations using S1P₄-HA (middle panel) or wild type HTC4 cells (right panel) are illustrated as control experiments. One representative histogram of three similar experiments is shown in each figure.

**FIGURE 4.** Attenuation of S1P₁ receptor phosphorylation by PKC inhibition. S1P₁-HA expressing HTC4 cells were incubated for 15 h at 37 °C with 10 μM PKC activator PMA, 10 nM FTY720, PMA, and FTY720 together, 50 μM PKC inhibitor myr-ΨPKC, 300 nM FTY720, 50 nM PKC inhibitor staurosporine, 100 nM calphostin C, and 100 nM GF109203X. A black line represents vehicle-treated control cells, and HTC4 wild type cells are shown as negative control (green line). Similar incubations using S1P₁-HA-expressing HTC4 cells were incubated for 15 h at 37 °C with 10 μM PMA, 10 nM FTY720, and both stimuli together, similar to Fig. 3, and with 50 μM myr-ΨPKC, 300 nM FTY720, 50 nM staurosporine, 100 nM calphostin C, and 100 nM GF109203X, similar to Fig. 2. After immunoprecipitation of the S1P₁-HA receptor with an anti-HA antibody, receptor phosphorylation was detected by Western blot using an anti-phosphothreonine antibody. The observed threonine phosphorylation of the S1P₁-HA receptor reflected the observed surface expression (Figs. 2 and 3). Treatment with the PKC activator PMA resulted in slightly increased receptor phosphorylation, whereas single and co-treatment with 10 nM FTY720 attenuated S1P₁ receptor phosphorylation compared with untreated control cells (Fig. 4). A higher concentration of FTY720 (300 nM) did not further enhance inhibition of receptor phosphorylation. The PKC inhibitors myr-ΨPKC, staurosporine, calphostin C, and GF109203X were all effective (Fig. 4).

**FTY720, but Not FTY-P, Inhibits PKC**—The influence of the PKC activator PMA on FTY720-induced down-regulation of S1P₁-HA surface expression pointed at a potential direct cellular interaction of FTY720 with PKC. Detection of PKC activity with the PepTag® fluorescent protein kinase assay revealed a potent inhibition of a mixture of predominantly conventional isoforms of PKC (33) by FTY720 (Fig. 5A). Comparable inhibition was not observed with FTY-P. The effective amount of FTY720 was highly dependent on the amount of added PKC activator phosphatidylserine (PS). PS added at concentrations of 50 and 500 μM maximally activated PKC, whereas lower concentrations were less effective (Fig. 5B). PKC inhibition of 500 μM FTY720 in the presence of 500 μM PS was similarly efficient as 50 μM FTY720 with 50 μM PS added (Fig. 5C). Therefore the ratio of PS to FTY720 was the critical determinant for efficient PKC inhibition rather than absolute FTY720 concentrations. A ratio of 1:1 PS:FTY720 proved to be maximally effective with noticeable PKC inhibition starting at a ratio of 10:1 (Fig. 5C). Ten PKC isoforms including the conventional PKC α, β₁, β₂, and γ, the novel PKC δ, ε, η, and θ, and the atypical PKC ζ and ι were tested with regard to FTY720-induced inhibition using an ELISA-based PKC activity assay kit. There was no significant isotype specificity for FTY720 among these PKC isoforms, demonstrating that FTY720 serves as a pan-PKC inhibitor (Fig. 5D). AAL(S), an inactive stereospecific isoform of FTY720 that is neither phosphorylated by sphingosine kinases nor inducing lymphopenia in rodents (17), showed a much lower potency for PKC inhibition than FTY720 (Fig. 5E). The activity of most PKC isoforms was less affected by AAL(S) than FTY720; inhibition, however, was obvious for conventional PKC β₂, novel PKC θ, and atypical PKC ι. PKC itself had no phosphorylating activity in nontransfected vehicle- and compound-treated HTC4 cells (Fig. 3).

Altered S1P₁ Receptor Phosphorylation—To test whether or not PKC activation or inhibition alters the phosphorylation state of the S1P₁ receptor, S1P₄-HA-expressing HTC4 cells were treated for 15 h with 10 μM PMA, 10 nM FTY720, and both stimuli together, similar to Fig. 3, and with 50 μM myr-ΨPKC, 300 nM FTY720, 50 nM staurosporine, 100 nM calphostin C, and 100 nM GF109203X, similar to Fig. 2. After immunoprecipitation of the S1P₁-HA receptor with an anti-HA antibody, receptor phosphorylation was detected by Western blot using an anti-phosphothreonine antibody. The observed threonine phosphorylation of the S1P₁-HA receptor reflected the observed surface expression (Figs. 2 and 3). Treatment with the PKC activator PMA resulted in slightly increased receptor phosphorylation, whereas single and co-treatment with 10 nM FTY720 attenuated S1P₁ receptor phosphorylation compared with untreated control cells (Fig. 4). A higher concentration of FTY720 (300 nM) did not further enhance inhibition of receptor phosphorylation. The PKC inhibitors myr-ΨPKC, staurosporine, calphostin C, and GF109203X were all effective (Fig. 4).

**PMA Partially Rescues S1P₁ Receptor Down-regulation by FTY720**—Because S1P₁-HA surface expression was decreased after treatment with several PKC inhibitors, the next step was to investigate the effect of the PKC activator PMA on FTY720-induced down-regulation of S1P₁-HA surface expression. Compared with control cells, treatment with 10 nM FTY720 already resulted in significant down-regulation of S1P₁-HA surface expression (Fig. 3). Simultaneous treatment of cells with 10 μM PMA partially prevented from FTY720-induced down-regulation of S1P₁-HA surface expression (Fig. 3). PMA treatment had no effect on S1P₄ receptor surface expression, and no differences in fluorescence intensity were observed and 100 nM GF109203X (Fig. 2B). Consistent with the PKC inhibitor myr-ΨPKC, none of the tested PKC inhibitors induced down-regulation of S1P₄ receptor surface expression or shifted the fluorescence signal in nonexpressing control cells (Fig. 2B).

**PMA Partially Rescues S1P₁ Receptor Down-regulation by FTY720**—Because S1P₁-HA surface expression was decreased after treatment with several different PKC inhibitors, the next step was to investigate the effect of the PKC activator PMA on FTY720-induced down-regulation of S1P₁-HA surface expression. Compared with control cells, treatment with 10 nM FTY720 already resulted in significant down-regulation of S1P₁-HA surface expression (Fig. 3). Simultaneous treatment of cells with 10 μM PMA partially prevented from FTY720-induced down-regulation of S1P₁-HA surface expression (Fig. 3). PMA treatment had no effect on S1P₄ receptor surface expression, and no differences in fluorescence intensity were observed and 100 nM GF109203X (Fig. 2B). Consistent with the PKC inhibitor myr-ΨPKC, none of the tested PKC inhibitors induced down-regulation of S1P₄ receptor surface expression or shifted the fluorescence signal in nonexpressing control cells (Fig. 2B).
on FTY720, and incubation of FTY720 with PKC did not result in FTY-P production (Fig. 5).

Similar Receptor Internalization Induced by S1P1 Activation and PKC Inhibition—Cyclical alteration of S1P1 receptor surface expression on T and B cells was suggested to be critical for regulation of lymphocyte circulation (3). S1P1 receptor internalization was therefore recognized as an important step in this process. Besides agonist-induced activation and internalization of the S1P1-HA receptor (green), and with 4′,6-diamidino-2′-phenylindole dihydrochloride (DAPI) for visualization of nuclei (blue), B, mouse splenocytes were stimulated for 15 h with 100 nM calphostin C (Col C) and immobilized antibodies against CD3 and CD28 (30 μg each) and for 1 h with 300 nM FTY720 and 1 μM S1P as indicated. Endogenous S1P1 receptor was detected in isolated nuclear cell fractions by Western blot using anti-S1P1 antibody. Expression of the nuclear protein histone deacetylase 1 (HDAC1) is shown as control (Cont.). Signals derived from two independent experiments were quantified with ImageJ 1.42q (National Institutes of Health). Shown are the means ± S.E.

FIGURE 5. PKC is blocked by FTY720, but not FTY-P. PKC activity was addressed with the PepTag fluorescent PKC assay as described under “Experimental Procedures.” A, representative UV-illuminated agarose gel with fluorescently labeled C1 peptide phosphates. FTY720 and FTY-P were added at a concentration of 500 μM. B, PS dependence of PepTag fluorescent PKC assay. C, inhibition of PKC by FTY720 in dependence of PS (500 μM, left panel; 50 μM, right panel). The reactions were performed with varying concentrations of FTY720 or vehicle as indicated. Relative enzyme activity was quantified by densitometric analysis of the fluorescence intensity of phosphorylated PKC substrate and is expressed as a percentage of PKC activity. D and E, influence of 10 μM FTY720 (D) or AAL(S) (E) on enzyme activity of different PKC-isotypes as indicated. PKC activity was quantified by ELISA as illustrated under “Experimental Procedures.” The results represent normalized PKC activity in percent (vehicle control was set to 100%). F, FTY720 and FTY-P concentrations after incubation with PKC, analyzed by LC/MS/MS. The results represent the means ± S.E. of three separate experiments in each figure.

FIGURE 6. Internalization of S1P1-HA by PKC inhibition. A, S1P1-HA-transfected HTC4 cells were stimulated for 1 h with 1 μM S1P and for 15 h with 300 nM FTY720 and 100 nM calphostin C as indicated. The cells were fixed, permeabilized, and stained with anti-HA antibody for expression and localization of the S1P1-HA receptor (green), and with 4′,6-diamidino-2′-phenylindole dihydrochloride (DAPI) for visualization of nuclei (blue). B, mouse splenocytes were stimulated for 15 h with 100 nM calphostin C (Col C) and immobilized antibodies against CD3 and CD28 (30 μg each) and for 1 h with 300 nM FTY720 and 1 μM S1P as indicated. Endogenous S1P1 receptor was detected in isolated nuclear cell fractions by Western blot using anti-S1P1 antibody. Expression of the nuclear protein histone deacetylase 1 (HDAC1) is shown as control (Cont.). Signals derived from two independent experiments were quantified with ImageJ 1.42q (National Institutes of Health). Shown are the means ± S.E.
stained for S1P₁ receptor expression and nuclear DNA. Confocal microscopy revealed that treatment with calphostin C, FTY720, and S1P resulted in efficient S1P₁ receptor internalization (Fig. 6A). No co-staining of the S1P₁ receptor and the nuclei was detected. To confirm this result in primary lymphocytes, mouse splenocytes were treated for 15 h with 100 nM calphostin C and cross-linked antibodies against CD3 and CD28 and for 1 h with 1 μM S1P and 300 nM FTY720. Western blots revealed similar amounts of S1P₁ receptor in nuclear splenocyte fractions after stimulation with anti-CD3 and anti-
CD28 antibodies, FTY720, or S1P, and stimulation with calphostin C in tendency resulted in lower nuclear S1P₁ signal, although these differences were not statistically significant (Fig. 6B). PKC inhibition thus internalized the S1P₁ receptor via the endocytic pathway similar to S1P and FTY-P without detectable translocation to the nuclear envelope.

**Cell-based and in Vivo Inhibition of PKC by FTY720—**To demonstrate the relevance of FTY720-induced PKC inhibition for S1P₁ receptor down-regulation and induction of lymphopenia, PKC activity was determined in S1P₁-HA-expressing HTC₄ cells treated with different concentrations of FTY720 and its stereoselective analogs AAL(R/S). Compared with vehicle-treated control cells, treatment with 10 nm FTY720 already resulted in considerable PKC inhibition (Fig. 7A). AAL(R) and particularly AAL(S) were noticeably less efficient PKC inhibitors than FTY720 in this cell-based assay, requiring concentrations of 100 nm to 1 nm for similar effectiveness (Fig. 7A). The PKC inhibitors staurosporine, calphostin C, and GF109203X were similarly or even less effective than FTY720 (Fig. 7A). PKC inhibition by FTY720 was also tested in vivo. C57BL/6 mice were treated orally for 3 days with 0.3, 1, and 3 mg/kg/day FTY720, and after their lymphoid organs, lymph nodes, spleen, and thymus were harvested, they were tested for PKC activity. Compared with untreated control mice, all lymphoid organs from FTY720-treated mice revealed decreased PKC activity (Fig. 7B). The loss of PKC activity was most dramatic in spleen, followed by lymph nodes and thymus. The onset of PKC inhibition was already seen with the lowest concentration of 0.3 mg/kg/day FTY720 (Fig. 7B). Treatment of mice with either 20 mg/kg/day DOP, which increased endogenous levels of S1P and Sph in lymphoid organs, or 3 mg/kg/day AAL(S) did not result in PKC inhibition (Fig. 7B).

**In Vivo Inhibition of S1P₁ Receptor Phosphorylation—**Similar to S1P₁-HA receptor phosphorylation in HTC₄ cells (Fig. 4), we also tested the phosphorylation state of the endogenous S1P₁ receptor in lymph nodes, spleen, and thymus from mice treated for 3 days with 1 or 3 mg/kg/day FTY720, 3 mg/kg/day AAL(S), and 20 mg/kg/day DOP. Treatment of mice with the highest concentration of FTY720 demonstrated much lower S1P₁ receptor phosphorylation in all examined lymphoid tissues compared with untreated control mice (Fig. 7C). Phosphorylation was not altered with the lower dose of 1 mg/kg/day FTY720. In line with the PKC activity measurements (Fig. 7B), treatment of mice with AAL(S) and DOP did not change the phosphorylation state of the S1P₁ receptor in lymph nodes, thymus, and spleen (Fig. 7D). PKC inhibition by FTY720 therefore resulted in reduced S1P₁ receptor phosphorylation in vivo, whereas AAL(S) and DOP were ineffective.

**Contribution of FTY720-induced PKC Inhibition to Lymphopenia Induction—**FTY720-induced lymphopenia may arise from its phosphorylation by SK2 to FTY-P and consequent S1P₁ receptor stimulation (30, 31) or by the herein described phosphorylation- and agonist-independent S1P₁ receptor down-regulation via PKC inhibition. To test the contribution of both mechanisms, SK2-deficient mice were treated with 3 mg/kg/day FTY720 and analyzed after 5 days for the onset of lymphopenia. PKC inhibition in lymphoid organs was evident under these conditions (Fig. 7B), and phosphorylation of FTY720 to FTY-P was less than 1% compared with wild type mice because of the lack of SK2 expression (Fig. 7E). Sph and S1P levels in plasma and lymphoid tissues were not considerably altered during FTY720 treatment (Fig. 7E). In comparison with wild type control mice, which developed a severe B and T cell lymphopenia, SK2-deficient mice only responded with a comparably mild phenotype (Fig. 7F). The share of FTY720-induced PKC inhibition and down-regulation in the onset of lymphopenia was therefore limited, and S1P₁ receptor agonism of FTY-P proved to be the principal mechanism of FTY720-induced lymphopenia. Attempts to use PKC inhibitors in vivo for the investigation of lymphopenia failed because of severe side effects and circulation-independent alterations of lymphocyte populations in blood and lymphoid tissues.

**DISCUSSION**

Two different mechanisms are currently used to explain the block of lymphocyte egress by FTY720: Down-regulation of the exit signal-sensing S1P₁ receptor on lymphocytes (1) and activation of S1P₁ on sinus-lining endothelial cells, resulting in the closure of suggested portals (14). Whereas the first mechanism basically relies on S1P₁ receptor inhibition, the latter proposes S1P₁ receptor activation as the critical event for lymphopenia induction. Evidence exists for both hypotheses: lymphocyte-specific S1P₁ receptor-deficient mouse mutants manifest a severe block in thymocyte and lymphocyte egress from thymus and lymph nodes, respectively, demonstrating the importance of S1P₁ receptor expression on thymocytes and patrolling lymphocytes for their exit from thymus and secondary lymphoid organs (1, 4). On the other hand S1P₁ receptor agonists prevent lymphocytes from crossing the lymphatic endothelium, which was blocked by otherwise inactive S1P₁ receptor antagonists (14, 39). Furthermore B cells seem to exit lymph nodes independently from S1P-mediated chemotaxis (40). The contribu-
*S1P*<sub>1</sub> Down-regulation by PKC Inhibition

...tion of both proposed mechanisms to FTY720-induced lymphopenia is therefore unclear. The presented data show that S1P<sub>1</sub> receptor surface expression can be abolished by PKC inhibition without S1P<sub>1</sub> receptor activation. In vivo inhibition of PKC, however, only induced a very mild lymphopenia (Fig. 7F), indicating that S1P<sub>1</sub> receptor stimulation mostly accounts for FTY720-induced egress inhibition, and S1P<sub>1</sub> receptor down-regulation is a secondary, less important event, at least in case of FTY720 treatment. This is in line with recent reports showing the requirement of FTY720 phosphorylation by SK2 for efficient lymphopenia at low FTY720 concentrations (30, 31) and the independence of B cell exit from S1P-mediated chemotaxis (40). It also explains the observation that S1P<sub>1</sub> receptor agonists and not antagonists were able to induce lymphopenia *in vivo*, although agonists could reverse lymphopenia induced by agonists (39). It should be noted here that the preferential stimulating activity of FTY-P over FTY720-induced S1P<sub>1</sub> receptor down-regulation does not exclude the imperative role of S1P<sub>1</sub> receptor cell surface expression for lymphocyte exit. But it is obvious from the presented studies that S1P<sub>1</sub> receptor down-regulation has to be rather complete to significantly affect lymphocyte egress. In contrast to S1P<sub>1</sub> receptor antagonists, agonists may be more potent either by establishing endothelial cell barriers (14) or by neutralizing a postulated gradient of the endogenous ligand S1P in lymphoid tissues (3).

The presented data demonstrate that modulation of S1P<sub>1</sub> receptor surface expression is possible without extracellular stimuli. Another agonist-independent mechanism for modulation of S1P<sub>1</sub> receptor surface expression is the observed direct interaction of S1P<sub>1</sub> with CD69, which prevents cell surface expression of both molecules when co-expressed (41). Transgenic expression of CD69 in T cells led to accumulation of mature T cells in thymus (42), resembling a similar phenotype to S1P<sub>1</sub> receptor deficiency (1, 4) and FTY720 treatment (43). PKC inhibition also leads to S1P<sub>1</sub> receptor down-regulation, and FTY720 itself is a potent PKC inhibitor (Figs. 2 and 5C). Although the major immunomodulatory activity at low nanomolar FTY720 concentrations derives mainly from its phosphorylated derivative and S1P<sub>1</sub> receptor agonist FTY-P (30, 31), mild lymphopenia in FTY720 phosphorylation-defective SK2-deficient animals after treatment with 3 mg/kg/day FTY720 can be attributed to FTY720-induced PKC inhibition and consequent down-regulation of the S1P<sub>1</sub> receptor on lymphocytes (Fig. 7, B and F). Watterson *et al.* (28) reported that PKC stimulation with PMA leads to S1P<sub>1</sub> receptor phosphorylation and subsequent internalization. These studies, however, focused on the immediate effects of PKC stimulation within minutes, whereas onset of S1P<sub>1</sub> receptor down-regulation by PKC inhibitors typically required several hours (Fig. 2). It is likely that PKC directly phosphorylates S1P<sub>1</sub> and induces immediate and transient receptor down-regulation and additionally alters receptor availability by targeting S1P<sub>1</sub> cell surface expression indirectly via phosphorylation of other signaling molecules with the observed delayed onset and prolonged efficacy for receptor internalization after PKC inhibition. The data from Watterson *et al.* (28) are therefore not contradictory to this study but highlight a different regulatory aspect.

Sph is able to accumulate in lymphocytes just like FTY720 (35). Preincubation of primary mouse splenocytes with Sph specifically inhibited chemotaxis to S1P (Fig. 1). Significant increases in S1P levels were not observed, and combined pre- and co-incubation of mouse splenocytes with the S1P-blocking anti-S1P antibody Sphingomab<sup>TM</sup> did not prevent inhibition of chemotaxis to S1P by Sph (Fig. 1). An alternative explanation for agonist-induced receptor down-regulation and inhibition of chemotaxis to S1P is the repression of PKC by Sph (25). The PKC-specific inhibitor myr-ΨPKC also induced partial down-regulation of the S1P<sub>1</sub> receptor (Fig. 2). But Sph levels typically do not reach the concentrations necessary for PKC inhibition, and treatment of mice with the S1P-lyase inhibitor DOP, which also induced Sph accumulation, did not inhibit PKC activity (Fig. 7B). PKC inhibition by Sph therefore does not seem to occur even after DOP treatment, possibly because of misplaced subcellular localization, whereas PKC inhibition by FTY720 was obvious after treatment (Fig. 7B).

Although no direct PKC phosphorylation sites are currently known for the S1P<sub>1</sub> receptor, computational analysis revealed Thr<sup>236</sup> and Thr<sup>257</sup> as putative PKC phosphorylation sites for both human and mouse S1P<sub>1</sub> receptors. In line with previous studies (28, 29), PKC inhibition resulted in attenuated S1P<sub>1</sub> receptor phosphorylation in S1P<sub>1</sub>-HA-transfected HTC4 cells (Fig. 4). Although S1P<sub>1</sub> receptor phosphorylation was only partially reduced in these overexpressing cells after addition of various PKC inhibitors, a pronounced defect in phosphorylation of the endogenous mouse S1P<sub>1</sub> receptor was observed in lymphoid tissues from mice treated with 3 mg/kg/day FTY720 for 3 days (Fig. 7C). High dosage of FTY720 therefore inhibits PKC and impairs S1P<sub>1</sub> receptor phosphorylation *in vivo*.

FTY720 was shown to directly affect several different cellular enzymes including PLA2 (22), S1P-lyase (23), SK1/2 (21), and ceramide synthases (24). But S1P-lyase inhibition was not evident in the *in vivo* setting (23), and possible contributions of PLA2, SK1/2, and ceramide synthase for the *in vivo* efficacy of FTY720 were not even tested (21, 22, 24). This study clearly identifies FTY720 as a pan-PKC inhibitor not only *in vitro* but also *in vivo* at physiologically relevant concentrations (Figs. 5, A–D, and 7, A and B). PKC inhibition resulted in S1P<sub>1</sub> receptor down-regulation (Fig. 2). Although the contribution of PKC inhibition to the onset of lymphopenia was minor compared with S1P<sub>1</sub> receptor agonism (Fig. 7F), it may explain observations linking FTY720 treatment to increased apoptosis (44), tumor suppression (45), and clearance of chronic viral infection (46). Vice versa, PKC inhibitors may affect lymphocyte circulation by altering S1P<sub>1</sub> receptor cell surface expression.

Acknowledgments—We thank Anika Münk for excellent technical assistance and Constantin Bode for performing LC/MS/MS measurements. We are grateful to Elisabeth Krenmer for the anti-HA antibody and to Roger Sabbadini for the anti-S1P antibody Sphingomab<sup>TM</sup>.

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