Embryonic fibroblasts from S1P (sphingosine-1-phosphate) lyase-deficient mice [Sgpl1−/− MEFs (mouse embryonic fibroblasts)] are characterized by intracellular accumulation of S1P, elevated cytosolic [Ca\(^{2+}\)], and enhanced Ca\(^{2+}\) storage. Since S1P, produced by sphingosine kinase 2 in the nucleus of MCF-7 cells, inhibited HDACs (histone deacetylases) [Hait, Allegood, Maceyka, Strub, Harikumar, Singh, Luo, Marmorstein, Kordula, Milstein et al. (2009) Science 325, 1254–1257], in the present study we analysed whether S1P accumulated in the nuclei of S1P lyase-deficient MEFs and caused HDAC inhibition. Interestingly, nuclear concentrations of S1P were disproportionately elevated in Sgpl1−/− MEFs. HDAC activity was reduced, acetylation of histone 3-Lys\(^{8}\) was increased and the HDAC-regulated gene p21 cyclin-dependent kinase inhibitor was up-regulated in these cells. Furthermore, the expression of HDAC1 and HDAC3 was reduced in Sgpl1−/− MEFs. In wild-type MEFs, acetylation of histone 3-Lys\(^{8}\) was increased by the S1P lyase inhibitor 4-deoxypyridoxine. The non-specific HDAC inhibitor trichostatin A elevated basal [Ca\(^{2+}\)], and enhanced Ca\(^{2+}\) storage, whereas the HDAC1/2/3 inhibitor MGCD0103 elevated basal [Ca\(^{2+}\)], without influence on Ca\(^{2+}\) storage in wild-type MEFs. Overexpression of HDAC1 or HDAC2 reduced the elevated basal [Ca\(^{2+}\)], in Sgpl1−/− MEFs. Taken together, S1P lyase-deficiency was associated with elevated nuclear S1P levels, reduced HDAC activity and down-regulation of HDAC isoenzymes. The decreased HDAC activity in turn contributed to the dysregulation of Ca\(^{2+}\) homoeostasis, particularly to the elevated basal [Ca\(^{2+}\)], in Sgpl1−/− MEFs.

Key words: Ca\(^{2+}\) signalling, class I histone deacetylase, histone acetylation, sphingosine-1-phosphate, sphingosine-1-phosphate lyase, trichostatin A.

INTRODUCTION

S1P (sphingosine-1-phosphate) is an important bioactive lipid that regulates growth, survival and migration of many cell types and is involved in diverse physiological and pathophysiological processes in the immune, cardiovascular and nervous systems [1–4]. S1P is produced from sphingosine by two isoforms of SphK (sphingosine kinase), SphK1 and SphK2, that are components of complex signalling pathways [5–7]. Degradation of S1P occurs either by dephosphorylation or by irreversible cleavage catalysed by S1P lyase [8,9]. The activity of extracellular S1P is mediated by five specific G-protein-coupled receptors, termed S1P\(_{1–5}\) (see [10] for an overview and nomenclature). G-protein-coupled S1P receptors are widely expressed and play a role in vertebrate development and tissue homoeostasis. They regulate, for example, angiogenesis, trafficking of immune cells, vascular tone and permeability, inflammation and cancer growth [11–14]. Interestingly, despite the multitude of effects mediated by the G-protein-coupled S1P receptors, S1P also has intracellular activities and target sites. Intracellular S1P has been implicated in stimulation of cell growth, protection from apoptosis and mobilization of Ca\(^{2+}\) from intracellular storage sites (reviewed in [4,15]). For example, S1P formed by SphK1 stimulated the growth of intestinal adenomas in Apc\(^{min}\) mice independently of G-protein-coupled S1P receptors [16]. Another example are mast cells from SphK2-deficient mice, which have reduced intracellular levels of S1P and a defective Ca\(^{2+}\) mobilization after IgE receptor cross-linking which could not be compensated by extracellular S1P [17]. In fact, the first paper showing that S1P was able to release Ca\(^{2+}\) from intracellular stores was published as early as 1994 [18]. Later, we demonstrated that intracellular S1P generated by photolysis of caged S1P mobilized Ca\(^{2+}\) from thapsigargin-sensitive stores independently of G-protein-coupled S1P receptors [19]. Also, in endothelial cells from human pulmonary artery, photolysis of caged S1P caused a pertussis-toxin-sensitive mobilization of stored Ca\(^{2+}\) and improvement of the vascular barrier function [20]. Since 2009, a number of intracellular target sites have been suggested, and this area of research is expanding rapidly [4]. Interestingly, the reported intracellular activities of S1P differ strongly with regard to target, subcellular localization and functional role. For example, it has been shown that S1P, formed by SphK2 in the nucleus, directly binds to HDACs (histone deacetylases), inhibits their activity and thereby regulates gene transcription [21]. S1P that was formed by SphK1 in the cytosol was able to act as a cofactor of the E3 ubiquitin ligase TRAF-2 [TNF (tumour-necrosis-factor)-receptor-associated factor 2], thereby mediating the activation of NF-κB (nuclear factor κB) by TNFα [22]. Mitochondrial S1P/SphK2, on the other hand, played a role in regulation of cytochrome c oxidase and the respiratory chain via interaction with prolinat-2 [23]. Finally, inhibition of VDAC1 (voltage-dependent anion channel 1) by S1P, as well as overexpression of S1P phosphate-1 or S1P lyase, caused inhibition of amyloid-β secretion, and S1P bound to and activated β-site amyloid precursor protein cleaving enzyme-1 [24]. However, the target that mediates the release of stored Ca\(^{2+}\) by intracellular S1P remains, until now, unknown. In the present study, we have further analysed the intracellular activities of S1P using embryonic fibroblasts from S1P...
lyase-deficient mice [Sgpl1−/−] MEFs (mouse embryonic fibroblasts), in which S1P accumulates [25]. Mice with a targeted deletion of S1P lyase have a reduced weight gain and usually die within 8 weeks [26]. They suffer from multiple organ defects, such as malformations of the vasculature and the skeleton, and a kidney dysfunction [26]. Another study has described significant lesions in histological sections of lung, heart, urinary tract and bone [27]. S1P lyase-deficient mice have elevated concentrations of S1P, sphingosine and/or ceramide in serum and tissues [27–29]. The disruption of the S1P concentration gradient which drives lymphocytes to emigrate from lymphoid tissues into lymph and blood leads to a severe lymphopaenia [27,28]. In our previous study with Sgpl1−/− MEFs, we observed an accumulation of S1P and sphingosine also in these cultured cells [25]. Sgpl1−/− MEFs were furthermore characterized by a disturbed Ca2++ augmented agonist-induced [Ca2+]i increases and enhanced storage of Ca2++ in thapsigargin-sensitive stores [25]. During the present investigation we observed that S1P was particularly high in nuclear preparations of Sgpl1−/− MEFs. Therefore we addressed the question of whether S1P, accumulating in the nuclei of S1P lyase-deficient cells, was able to inhibit HDACs, as it had been shown for nuclear S1P produced by Sphk2 [21]. We demonstrate in the present study that not only HDAC activity, but also the expression of HDAC isoenzymes was reduced in S1P lyase-deficient MEFs. Furthermore, we show that HDAC1 and HDAC2 regulate basal [Ca2+]i, in these cells and thus demonstrate that HDACs contribute to the regulation of Ca2++ homeostasis.

EXPERIMENTAL

Materials

Trichostatin A and Nonidet P40 were purchased from Calbiochem/Merck Biosciences. DOP (4-deoxypyridoxine) was from Sigma and MGCD0103 was from Selleck Chemicals LLC. The standard lipids for MS, d-erythro-C17-S1P and d-erythro-C17-sphingosine were from Avanti Polar Lipids. All other chemicals were from previously described sources [25,30].

Cell culture and transfection

Embryonic fibroblasts from S1P lyase-deficient and wild-type mice were provided by Dr Paul P. Van Veldhoven (KU Leuven, Leuven, Belgium). Cells were cultured in DMEM (Dulbecco’s modified Eagle’s medium)/Ham’s F12 supplemented with 100 units/ml penicillin G, 0.1 mg/ml streptomycin and 10% FBS (fetal bovine serum) in a humidified atmosphere of 5% CO2/95% air at 37°C. Before experiments, the cells were kept in serum-free medium overnight. Pre-treatment with trichostatin A and MGCD0103 was performed for 16 h unless otherwise stated. For inhibition of S1P lyase, the cells were kept for 7 days in medium containing 4 mM DOP.

The plasmid for expression of S1P lyase N-terminally tagged with YFP (yellow fluorescent protein) was from Source BioScience LifeSciences [ORF (open reading frame) expression clone IOH28907 in pDEYFP-C1amp vector]. The insert has a silent mutation in position 564 (ATT instead of ATC), which does not result in amino acid exchange. MEFs were transfected with 5 μg of plasmid DNA per 145-mm dish with the Jet Prime transfection reagent (Peqlab) according to the manufacturer’s instructions, and used for experiments after 24 h. Transfection efficiency was ~10% as monitored by fluorescence microscopy. FLAG-tagged HDAC1 in the pBJ1 vector was a gift from Dr Stuart L. Schreiber (Howard Hughes Medical Institute, Cambridge, MA, U.S.A.). FLAG-tagged HDAC2 was provided by Dr Ed Seto (H. Lee Moffitt Cancer Center & Research Institute, Tampa, FL, U.S.A.). These constructs were transfected into MEFs by electroporation using the Amaxa Nucleofector II device and the Amaxa Nucleofector Kit V (Lonza). Briefly, 2×10^6 cells suspended in Nucleofector solution V were mixed with 10 μg of plasmid DNA and electroporated using program A-23. Afterwards, the cells were seeded on to 145-mm dishes and cultivated for 3 days in serum-containing medium before they were serum-starved overnight and used in the experiments. Transfection efficiency was ~10% as estimated from parallel transfections with the pEGFP-C1 vector leading to expression of GFP (green fluorescent protein).

Measurement of HDAC activity

For preparation of crude nuclear extracts, serum-starved MEFs grown on 145-mm dishes were washed with ice-cold PBS and scraped into 1 ml of PBS supplemented with 0.1 mM EDTA. All of the following steps were performed at 4°C. After centrifugation for 1 min at 2400 g, the pellets were resuspended in 300 μl of buffer 1 [10 mM Hepes, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol and 0.5 mM PMSF (pH 7.9)]. The samples were incubated for 15 min on ice before 20 μl of 10% Nonidet P40 was added. For centrifugation for 1 min at 16200 g, the pellets were resuspended in 70 μl of buffer 2 [20 mM Hepes, 25% glycerol, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and 0.5 mM PMSF (pH 7.9)]. The samples were shaken for 20 min at 4°C and centrifuged for 20 min at 16200 g. The supernatants containing the nuclear preparations were collected and stored at −80°C until use. HDAC activity was determined using a fluorimetric HDAC activity assay kit (Merck Biosciences). Briefly, the deacetylation reaction was performed with 0.21 mg/ml protein for 30 min at room temperature (21°C). Then, the developer was added, the samples were incubated for an additional 10 min, and the fluorescence was measured with 360 nm excitation and 480 nm emission wavelengths.

Lipid extractions

Serum-starved MEFs grown on 145-mm dishes were washed with ice-cold PBS and scraped into 1 ml of PBS supplemented with 0.1 mM EDTA. All of the following steps were performed at 4°C. After centrifugation for 1 min at 1800 g and 4°C. The pellets were resuspended in 350 μl of buffer 1 with 10 mM Hepes, 10 mM KCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and 0.5 mM PMSF (pH 7.9). The samples were centrifuged for 10 min at 8000 g and 4°C. The pellets were resuspended in 500 μl of buffer 2 with 10 mM Hepes, 10 mM KCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and 0.5 mM PMSF (pH 7.9). The samples were sonicated and centrifuged for 4°C and centrifuged for 10 min at 68 g. The aqueous phases containing the nuclear preparations were collected and stored at −80°C until use. HDAC activity was determined using a fluorimetric HDAC activity assay kit (Merck Biosciences). Briefly, the deacetylation reaction was performed with 0.21 mg/ml protein for 30 min at room temperature (21°C). Then, the developer was added, the samples were incubated for an additional 10 min, and the fluorescence was measured with 360 nm excitation and 480 nm emission wavelengths.

Western blot analysis

Cell lysates were separated by SDS/PAGE and blotted on to PVDF membranes. Blots were stained with antibodies directed against
acetylated H3K9 (histone 3-Lys9), acetylated H4K5 (histone 4-Lys5), HDAC1, HDAC2, HDAC3 (Cell Signalling Technology) or β-actin (Santa Cruz Biotechnology), and analysed with HRP (horseradish peroxidase)-conjugated secondary antibodies using the ECL (enhanced chemiluminescence) system (GE Healthcare).

Quantitative real-time PCR

RNA was isolated from serum-starved MEFs with TRIzol® (Sigma–Aldrich). cDNA was prepared with the RevertAid first-strand cDNA synthesis kit (Fermentas). Real-time PCR was performed with the Applied Biosystems 7500 Fast Real-Time PCR System. Probes, primers and the reporter dyes 6-FAM (6-carboxyfluorescein) and VIC® were from Applied Biosystems. The cycling conditions were 95 °C for 15 min (1 cycle), followed by 95 °C for 15 s and 60 °C for 1 min (40 cycles). mRNA levels were analysed by the ΔΔCt method with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as a reference.

[Ca2+]i measurements

[Ca2+], was measured in fura 2-loaded MEFs as described previously [25]. Briefly, the cells were detached with trypsin, resuspended in Hanks buffered salt solution [118 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 5 mM D-glucose and 15 mM Hepes (pH 7.4)] and loaded with 1 μM fura 2/AM (fura 2 acetoxymethyl ester) for 45 min at room temperature. The measurements were performed in a Hitachi F2500 spectrofluorometer at room temperature. The excitation switched between 340 nm and 380 nm, whereas the emission was recorded at 510 nm. After determination of maximum and minimum fluorescence, [Ca2+], was calculated according to Grynkiewicz et al. [32].

Data analysis and presentation

Results are means ± S.D. from a representative experiment performed with the indicated number (n) of replicates, or means ± S.E.M. from the indicated number (n) of independent experiments. Data were assessed for significance by one-way ANOVA followed by Bonferroni’s post hoc test, or by Student’s t test, as indicated, using the GraphPad Prism program (* P < 0.05; ** P < 0.01; *** P < 0.001; n.s., not significant).

RESULTS

Nuclear accumulation of S1P in S1P lyase-deficient MEFs

We had observed previously that both sphingosine and S1P accumulated in MEFs from S1P lyase-deficient mice [25]. In whole-cell lysates, the concentration of sphingosine was ~8 pmol/mg of protein in wild-type and ~15 pmol/mg of protein in knockout MEFs, and the concentration of S1P was ~0.16 pmol/mg of protein in wild-type and ~0.8 pmol/mg of protein in knockout MEFs [25]. Since S1P, produced by SphK2 in the nucleus of MCF-7 breast cancer cells, was able to inhibit HDACs [21], we wondered whether S1P was elevated in the nuclei of S1P lyase-deficient cells. Interestingly, the concentrations of sphingosine and S1P were higher in the nuclear preparations than in cell lysates. Nuclear sphingosine amounted to ~170 pmol/mg of protein in both cell types, whereas nuclear S1P amounted to ~0.42 pmol/mg of protein in wild-type and ~22 pmol/mg of protein in S1P lyase-deficient MEFs (Figure 1). Thus S1P accumulated disproportionally in the nuclei of S1P lyase-deficient MEFs. In comparison, Hait et al. [21] measured S1P concentrations of 4.7 pmol/mg of protein in nuclei of vector-transfected and ~28 pmol/mg of protein in nuclei of SphK2-overexpressing MCF-7 cells. Thus the concentration of S1P in the nuclei of S1P lyase-deficient MEFs was within the range in which HDAC inhibition had been observed.

Reduced HDAC activity and down-regulation of HDAC isoenzymes in S1P lyase-deficient MEFs

It could be argued that the high nuclear S1P in S1P lyase-knockout MEFs does not cause HDAC inhibition because it originates from a different source than in the investigation of Hait et al. [21]. In order to clarify this, we analysed histone acetylation and HDAC activity in our cells. Hait et al. [21] had shown that S1P promoted (i) the acetylation of H3K9, but not acetylation of histone 3-Lys14, (ii) the acetylation of H4K5 and (iii) weakly, the acetylation of histone-2B at Lys12 [21]. We therefore focused on H3K9 and H4K5 and observed that acetylation of H3K9, but not H4K5, was significantly enhanced in lysates of S1P lyase-deficient cells (Figure 2A). Interestingly, cultivation of wild-type MEFs for 7 days in the presence of DOP, which inhibits pyridoxal phosphate-dependent enzymes including S1P lyase, similarly enhanced the acetylation of H3K9 (Figure 2B). In the nuclear preparations of S1P lyase-deficient cells, HDAC activity was significantly reduced (Figure 2C), suggesting that HDACs, and not histone acetyltransferases, were involved in the altered histone acetylation in these cells. Finally, mRNA expression of p21 cyclin-dependent kinase inhibitor, which is a HDAC-regulated gene [33], was strongly (>50-fold) up-regulated in S1P lyase-deficient MEFs, to an extent that was approximately comparable with that induced by the non-specific HDAC inhibitor trichostatin A in wild-type cells (Figure 2D). We furthermore analysed the protein expression of class I HDACs and observed that HDAC1 and HDAC3, but not HDAC2, were significantly down-regulated in S1P lyase-deficient MEFs (Figure 3).

Contribution of the reduced HDAC activity to dysregulation of Ca2+ homoeostasis in S1P lyase-deficient MEFs

Next, we analysed whether the reduced HDAC activity was involved in the previously described dysregulation of Ca2+ homoeostasis [25]. In S1P lyase-deficient MEFs, basal [Ca2+]i, was elevated, [Ca2+]i, increases by agonists were augmented and Ca2+ storage in thapsigargin-sensitive stores was enhanced [25] (Figure 4). First we analysed the effect of re-expression of S1P lyase. In this series of experiments, only small amounts of plasmid DNA (i.e. 5 μg per 145-mm dish) were transfected for
Figure 2  Decreased HDAC activity and enhanced histone acetylation in S1P lyase-deficient MEFs

(A) Histone acetylation was analysed by Western blotting of MEF cell lysates with antibodies directed against acetylated H3K9 and H4K5. Shown are representative blots that were re-probed with anti-β-actin. The quantification of acetylated H3K9 (H3K9ac) was performed by densitometric analysis, comparing Sgpl1+/− with Sgpl1−/− MEFs after normalization to β-actin (means ± S.E.M.; n = 6; Student’s t test). (B) Acetylation of H3K9 was analysed in lysates of wild-type MEFs treated without or with 4 mM DOP for 7 days. Shown is a representative blot that was re-probed with anti-β-actin, and a quantification of acetylated H3K9 as described above (means ± S.E.M.; n = 3, Student’s t test). (C) HDAC activity was measured in the presence and absence of 5 mM trichostatin A (TSA) in nuclear preparations of Sgpl1+/− and Sgpl1−/− MEFs (means ± S.E.M.; n = 4; one-way ANOVA/Bonferroni’s post hoc test). (D) mRNA levels of p21 cyclin-dependent kinase inhibitor were determined by quantitative real-time PCR in Sgpl1−/− and Sgpl1+/− MEFs treated with vehicle or 1 μM trichostatin A (TSA) for 16 h (means ± S.E.M.; n = 5; one-way ANOVA/Bonferroni’s post hoc test). The Western blot shows the effect of trichostatin A (1 μM, 16 h) on H3K9 acetylation.

Figure 3  Reduced expression of HDAC isoenzymes in S1P lyase-deficient MEFs

(A) Expression of HDAC1, HDAC2 and HDAC3 was analysed in MEF cell lysates by Western blotting with specific antibodies. Shown are representative blots and the densitometric analysis comparing Sgpl1−/− with Sgpl1+/− MEFs after normalization to β-actin (means ± S.E.M.; n = 6; Student’s t test).

Figure 4  Decrease in elevated basal [Ca2+]i and enhanced Ca2+ storage in S1P lyase-deficient MEFs by re-expression of S1P lyase

[Ca2+]i was measured in wild-type (Sgpl1+/−) and S1P lyase-deficient (Sgpl1−/−) MEFs loaded with fura 2. The cells had been transfected with YFP or YFP-tagged S1P lyase (YFP-SPL) for ~24 h. Top panel, typical time courses of [Ca2+]i, before and after stimulation with 1 μM thapsigargin (means ± S.D. from a representative experiment; n = 3). Bottom panels, quantification of basal [Ca2+]i and thapsigargin-induced [Ca2+]i, increases, expressed as a percentage of Sgpl1+/− (means ± S.E.M. from three independent experiments; one-way ANOVA/Bonferroni’s post hoc test).

A short period of time (24 h), since a stronger expression of S1P lyase induced apoptosis in many of the cells (results not shown). Even this slight expression of S1P lyase led to a significant reduction of the elevated basal [Ca2+]i, and the enhanced Ca2+ storage (Figure 4), confirming the causal relationship between the knockout of S1P lyase and the dysregulated Ca2+ homoeostasis. Next, we analysed the effect of the non-specific HDAC inhibitor trichostatin A. Incubation of wild-type MEFs for 16 h with 1 μM trichostatin A caused an elevation of basal [Ca2+]i, from approximately 100 nM up to approximately 250 nM and thus up to values that were measured in S1P lyase-deficient MEFs (Figure 5). Furthermore, treatment of wild-type MEFs for 16 h with 1 μM trichostatin A augmented [Ca2+]i, increases induced by the SERCA (sarcoplasmic/endoplasmic reticulum Ca2+-ATPase) inhibitor thapsigargin from ~190 nM up to ~330 nM (Figure 5). In S1P lyase-deficient MEFs, however, thapsigargin-induced [Ca2+]i, increases were ~830 nM and thus even higher. In S1P lyase-deficient MEFs, trichostatin A further elevated basal [Ca2+]i, but slightly reduced thapsigargin-induced [Ca2+]i, increases (Figure 5). Importantly, a short incubation (30 min) with trichostatin A had no influence on cellular Ca2+ homoeostasis (Figure 6), indicating that trichostatin A did not directly interfere with Ca2+ signalling. We furthermore analysed the influence of MGCD0103, which inhibits purified HDAC1, HDAC2 and HDAC3 with IC50 values of 30–150 nM, 30–300 nM and 1–2 μM respectively, whereas it has no influence on HDAC isoforms 4/5/6/7/8 up to 10 μM [34,35]. As shown in Figure 7, treatment
Link between HDACs and Ca\(^{2+}\) homeostasis in S1P lyase-deficient MEFs

**Figure 5** Elevation of basal [Ca\(^{2+}\)] and increase in Ca\(^{2+}\) storage induced by long-term treatment of wild-type MEFs with trichostatin A

Wild-type (Sgpl1\(^{+/+}\)) and S1P lyase-deficient (Sgpl1\(^{-/-}\)) MEFs had been treated with vehicle or 1 μM trichostatin A (TSA) for 16 h. [Ca\(^{2+}\)] was measured with fura 2. The traces shown in the top panels are triplicates from a representative experiment (means ± S.D.), whereas the data in the histograms result from evaluation of four independent experiments each performed with \(n = 34\) (means ± S.E.M.; Student’s t test).

with MGCD0103 for 16 h significantly elevated basal [Ca\(^{2+}\)], in wild-type MEFs. Interestingly, MGCD0103 showed a biphasic concentration–response relationship, as 0.3–3 μM MGCD0103 elevated basal [Ca\(^{2+}\)] by approximately 2-fold, whereas 10 μM MGCD0103 further enhanced basal [Ca\(^{2+}\)], up to approximately 3-fold (Figure 7). Taking into account that the compound is probably less potent in intact cells than with purified enzymes, this result may indicate that the [Ca\(^{2+}\)] increase induced by 0.3–3 μM MGCD0103 is caused by inhibition of HDAC1 and/or HDAC2, whereas the additional effect at 10 μM is caused by inhibition of HDAC3. Thapsigargin-induced [Ca\(^{2+}\)] increases were not affected by MGCD0103 (Figure 7). Finally, we analysed whether the overexpression of HDAC1 or HDAC2 had an influence on Ca\(^{2+}\) homeostasis. Indeed, overexpression of either HDAC1 or HDAC2 caused a significant reduction of basal [Ca\(^{2+}\)], in S1P lyase-deficient MEFs (Figure 8). The HDACs did not fully restore the values that were observed in wild-type cells, probably because the transfection efficiency in the MEFs was rather low. Neither HDAC1 nor HDAC2 had a significant influence on Ca\(^{2+}\) storage (Figure 8), which matches the results with MGCD0103. Taken together, these data show that HDAC1 and HDAC2 regulate basal [Ca\(^{2+}\)], and that their reduced activity and/or expression contribute to the elevated basal [Ca\(^{2+}\)], in S1P lyase-deficient MEFs. The enhanced Ca\(^{2+}\) storage in S1P lyase-deficient MEFs and in trichostatin A-treated wild-type MEFs, however, cannot be explained by effects on HDAC1, HDAC2 or HDAC3.

**DISCUSSION**

Over the last few years, a number of intracellular target sites of S1P have been suggested that are remarkably different with regard to structure, subcellular localization and functional context [4]. A common theme seems to be the direct delivery of S1P to its targets by one of the two SphKs in a highly compartmentalized manner. Therefore the subcellular localization of S1P appears to determine its activity. On this background it is highly interesting that we observed high concentrations of S1P and sphingosine in the nuclei of wild-type MEFs and a disproportional accumulation of S1P in the nuclei of S1P lyase-deficient MEFs, with an ~6-fold increase in total cellular S1P and an ~40-fold increase in nuclear S1P in these cells. It remains an open question why S1P accumulates particularly in the nuclei of S1P lyase-deficient MEFs, since S1P lyase is attached to the ER (endoplasmic reticulum) with the catalytic domain extending into the cytosol.
Figure 8  Decrease in elevated basal $[\text{Ca}^{2+}]_i$, by overexpression of HDAC1 and HDAC2 in S1P lyase-deficient MEFs

$[\text{Ca}^{2+}]_i$ was measured in fura 2-loaded MEFs. Sgp1l$^{−/−}$ MEFs had been transfected with GFP, HDAC1 or HDAC2 for $\sim$96 h. Results from GFP-transfected Sgp1l$^{+/+}$ MEFs are shown for comparison. Basal $[\text{Ca}^{2+}]_i$ and thapsigargin-induced increases in $[\text{Ca}^{2+}]_i$ are expressed as a percentage of GFP-transfected Sgp1l$^{−/−}$ MEFs (means $\pm$ S.E.M.; $n=3$; one-way ANOVA/Dunnett's post hoc test).

[36,37] and therefore should preferentially degrade cytosolic S1P. However, S1P lyase also regulates extracellular S1P as it keeps S1P concentrations low in lymphatic tissues [38]. These observations suggest that S1P is transported not only across the plasma membrane, e.g. by the spinster-2 protein [39], but also within the cell, probably by intracellular transport proteins that shuttle S1P between subcellular compartments. Such a protein has been described for ceramide [40], but not yet for S1P.

Nuclear S1P has been linked to HDAC inhibition by the work of Hait et al. [21]. These authors have demonstrated that SphK2, which is found in the nucleus of several cell types, including MCF-7 breast cancer cells, was associated with HDAC1 and HDAC2 in repressor complexes, e.g. at the promoter of p21 cyclin-dependent kinase inhibitor, and enhanced histone 3 acetylation as well as transcription in these cells. Moreover, S1P directly bound to HDAC1 and HDAC2, but not to HDACs 3–8 or the class III HDAC SIRT1 (sirtuin 1) and inhibited their activity [21]. However, an independent confirmation of these data is still awaited. Using a different model system, we show in the present study that HDAC inhibition occurs in S1P lyase-deficient MEFs containing high nuclear concentrations of S1P: nuclear HDAC activity was reduced, histone acetylation was enhanced and p21 cyclin-dependent kinase inhibitor was up-regulated in S1P lyase-deficient cells. p21 (also named p21cip1/waf1) is a tumorigenic role for p21 has been observed in certain cellular contexts, since it can suppress apoptosis and even promote cell-cycle progression (discussed in [47,48]). Even a tumorigenic role for p21 has been observed in certain cellular contexts, since it can suppress apoptosis and even promote cell-cycle progression (discussed in [47,48]).

p21, on the other hand, does not always induce cell-cycle arrest, but has a number of additional roles, especially when localized in the cytosol [48]. However, cancer cells are much more sensitive to HDAC inhibitors than non-transformed cells, and the reason for this remains a matter of debate [42,45]. In normal cells, class I HDACs often regulate processes of differentiation and tissue-specific functions; for example, HDAC1 and HDAC2 regulate cardiac morphogenesis, growth and contractility [46] or control the transcriptional programme of myelination and the survival of Schwann cells [47]. p21, on the other hand, does not always induce cell-cycle arrest, but has a number of additional roles, especially when localized in the cytosol [48]. Even a tumorigenic role for p21 has been observed in certain cellular contexts, since it can suppress apoptosis and even promote cell-cycle progression (discussed in [49,50]).

Moreover, p21 protein expression is regulated by multiple post-transcriptional and post-translational mechanisms [50], and the up-regulation of p21 protein in S1P lyase-deficient MEFs remains to be shown.

In the present study, we focused on the dysregulation of Ca$^{2+}$ homoeostasis that we have observed earlier in S1P lyase-deficient MEFs [25], and provide a link between HDAC inhibition and high resting [Ca$^{2+}$], in these cells. First, basal [Ca$^{2+}$], in wild-type MEFs was strongly and significantly elevated by the non-selective HDAC inhibitor trichostatin A and by the HDAC1/2/3 inhibitor MGCD0103. Secondly, the elevated basal [Ca$^{2+}$], in S1P lyase-deficient MEFs was significantly reduced by overexpression of HDAC1 or HDAC2. These data indicate that class I HDACs regulate resting [Ca$^{2+}$], in MEFs, which to our knowledge has not been shown before, and that the reduced HDAC activity in S1P lyase-deficient MEFs contributes to the elevated basal [Ca$^{2+}$], in these cells. The Ca$^{2+}$ content of thapsigargin-sensitive stores was less affected by HDACs. First, although treatment with trichostatin A significantly enhanced Ca$^{2+}$ storage in wild-type MEFs, it did not increase Ca$^{2+}$ storage up to the high levels that were observed in S1P lyase-deficient MEFs. Secondly, thapsigargin-induced [Ca$^{2+}$], increases were not altered by MGCD0103 in wild-type MEFs or by overexpression of HDAC1 or HDAC2 in S1P lyase-deficient MEFs. Therefore Ca$^{2+}$ storage might be under the influence of trichostatin A-sensitive HDAC isoforms other than HDAC1/2/3 or regulated by a different mechanism. There are only few papers that show an influence of HDACs or HDAC inhibitors on Ca$^{2+}$ homoeostasis or on the proteins by which it is regulated.
Recently, it was shown that trichostatin A inhibited [Ca\(^{2+}\)] from endoplasmic reticulum-resident, integral membrane protein with the pyridoxal phosphate binding domain exposed to the cytosol. Biochem. Biophys. Res. Commun. 374, 413–428


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