Sphingosine 1-phosphate and human ether-a'-go-go-related gene potassium channels modulate migration in human anaplastic thyroid cancer cells

Muhammad Yasir Asghar¹, Tero Viitanen²,³, Kati Kemppainen¹ and Kid Törnquist¹,²

¹Department of Biosciences, Åbo Akademi University, Biocity, Artillerigatan 6, 20520 Turku, Finland
²The Minerva Foundation Institute for Medical Research, Biomedicum Helsinki, 00290 Helsinki, Finland
³Institute of Biomedicine/Physiology, University of Helsinki, PO Box 63, 00014 Helsinki, Finland

(Correspondence should be addressed to K Törnquist at Department of Biosciences, Åbo Akademi University; Email: ktornqvi@abo.fi)

Abstract

Anaplastic thyroid cancer (ATC) is the most aggressive form of human thyroid cancer, lacking any effective treatment. Sphingosine 1-phosphate (S1P) receptors and human ether-a'-go-go-related gene (HERG (KCNH2)) potassium channels are important modulators of cell migration. In this study, we have shown that the S1P₁–₃ receptors are expressed in C643 and THJ-16T human ATC cell lines, both at mRNA and protein level. S1P inhibited migration of these cells and of follicular FTC-133 thyroid cancer cells. Using the S1P₁,₃ inhibitor VPC-23019, the S1P₂ inhibitor JTE-013, and the S1P₂ receptor siRNA, we showed that the effect was mediated through S1P₂. Treatment of the cells with the Rho inhibitor C3 transferase abolished the effect of S1P on migration. S1P attenuated Rac activity, and inhibiting Rac decreased migration. Sphingosine kinase inhibitor enhanced basal migration of cells, and addition of exogenous S1P inhibited migration. C643 cells expressed a nonconducting HERG protein, and S1P decreased HERG protein expression. The HERG blocker E-4031 decreased migration. Interestingly, downregulating HERG protein with siRNA decreased the basal migration. In experiments using HEK cells overexpressing HERG, we showed that S1P decreased channel protein expression and current and that S1P attenuated migration of the cells. We conclude that S1P attenuates migration of C643 ATC cells by activating S1P₂ and the Rho pathway. The attenuated migration is also, in part, dependent on a S1P-induced decrease of HERG protein.

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Introduction

Anaplastic thyroid cancer (ATC) is the most aggressive form of human thyroid cancer. Due to its high invasive potential, mainly to lungs, brain, and bones, the patient has an average life span of 3–5 months after diagnosis (Nagaiah et al. 2011). Currently, there is no effective treatment available (Smallridge et al. 2009). Sphingosine 1-phosphate (S1P) can bind to five different receptors, denominated S1P₁–₅ (Ishii et al. 2002, 2004, Spiegel et al. 2002). S1P₁ and S1P₃, by activating the PI3K–Akt and Rac pathways, promote cell migration and a more invasive phenotype (Gonda et al. 1999, Windh et al. 1999, Okamoto et al. 2000, Ishii et al. 2002). In contrast, S1P₂ may activate the Rho and ROCK pathways, thus attenuating migration (Ishii et al. 2002, Hashimoto et al. 2008, Takashima et al. 2008, Takuwa et al. 2011, Witt et al. 2012). S1P₂ may also activate
phosphatase and tensin homolog (PTEN), a tumor suppressor gene, and, through inhibition of Akt, block cell proliferation and migration (Sanchez et al. 2005).

Several ion channels, including human ether a’-go-go-related gene (hKhV1.1, HERG (KCNH2)) potassium channel, are important regulators of cell migration and proliferation, especially in cancer (Arcangeli 2005, Prevarskaya et al. 2010). Interestingly, also nonconducting HERG channel proteins regulate cell proliferation and migration (Kaczmarek 2006). The possible importance of HERG in thyroid cancer is not known.

We have previously investigated the importance of S1P in modulating thyroid cancer cell migration and proliferation (Balzasar et al. 2006, Bergelin et al. 2009). These studies showed clearly that S1P potently enhanced migration of the thyroid follicular ML-1 cancer cells. However, later investigations revealed that the origin of several of the cell lines we studied was unclear (Schweppe et al. 2008). Thus, we wanted to investigate the effects of S1P on migration of verified ATC cell lines. In the present investigation, we have studied the expression of S1P1–3 and the importance of S1P to regulate the migration of human ATC C643 and THJ-16T cell lines and for comparison, the follicular FTC-133 thyroid cancer cell line. We show that all three receptors are expressed in these cells and that S1P potently inhibits the migration, apparently by activating S1P2. Furthermore, we also show that C643 cells express HERG channels, but in a nonconducting form. However, blocking HERG attenuated C643 cell migration. In addition, S1P transiently decreased the expression of HERG protein.

In control experiments using human embryonal kidney (HEK) cells overexpressing HERG, S1P also inhibited migration, evoked a transient decrease in HERG expression, and decreased the conductance of the HERG channel.

Materials and methods

Materials

DMEM, fatty acid-free (FAF)-BSA, PD 118057, poly-L-lysine, sodium pyruvate solution (1 mM), 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), and Mowiol 4-88 were purchased from Sigma–Aldrich. RPMI 1640 medium (without L-glutamine) was from Lonza (Basel, Switzerland). Fetal bovine serum (FBS), 2.5% trypsin, L-glutamine, penicillin/streptomycin, nonessential amino acids, Opti Mem, and F-12 (Ham’s nutrient medium) were from Gibco. S1P was from Biomol (Plymouth, PA, USA). JTE-013 was from Tocris Biosciences (Ellisville, MO, USA) and VPC23019 from Avanti Polar Lipids (Alabaster, AL, USA). Y-27632, Rac inhibitor (Raci), sphingosine kinase inhibitor (SKi), and the PTEN inhibitor potassium bisperoxo(1,10-phenanthroline)oxovanadate(V) [bpV(phen)] were from Calbiochem (San Diego, CA, USA). C3 transferase was purchased from Cytoskeleton, Inc. (Denver, CO, USA). Anti-hk, 11.1 (HERG) antibody and E-4031 were from Alomone Labs (Jerusalem, Israel). The Alexa Flour-568 goat anti-rabbit antibody was obtained from Invitrogen Detection Technologies. S1P1, S1P2, S1P3, and Hsc70 primary antibodies were from Santa Cruz Bio-technology, Inc. (Santa Cruz, CA, USA). HRP-conjugated goat anti-rabbit IgG and the Aurum total RNA isolation kit were from Bio-Rad Laboratories. HRP-conjugated anti-rat and anti-mouse IgG were from Cell Signaling Technology (Denver, MA, USA). Small interfering RNA (siRNA) for S1P1 and S1P3 as well as a scrambled control siRNA were purchased from Dharmacon, Inc. (Lafayette, CO, USA). S1P2 siRNA and another scrambled control siRNA were also obtained from Ambion (Austin, TX, USA). Superscript III reverse transcriptase and thyroid cDNA from normal human adult tissue were purchased from Invitrogen. PCR primers were synthesized by TAG (Copenhagen, Denmark) and the Ribogreen RNA quantification reagent was purchased from Molecular Probes (Eugene, OR, USA). Cell culture plastic ware and human collagen type I and type IV were from Becton Dickinson Biosciences (Bedford, MA, USA) and Transwell inserts for migration assays from Coring, Inc. (Corning, NY, USA). Bicinchoninic acid Protein Assay Reagent kit was from Pierce Biotechnology (Rockford, IL, USA). The Rac 1,2,3 G-LISA Activation Assay kit was from Cytoskeleton, Inc. All the chemicals and reagents used were of molecular biology and reagent grades.

Methods

Cell culture

The C643 ATC cells provided by Dr Nils-Erik Heldin (Karolinska Institute, Stockholm, Sweden) were cultured in DMEM supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. The ATC THJ-16T cells were a kind gift from Dr John Copland (Mayo Clinic, Jacksonville, FL, USA). The cells were cultured in RPMI 1640 supplemented with 10% FBS, 1% penicillin/streptomycin, 1 mM sodium pyruvate, and 25.03 mM HEPES. Wild-type HEK-293 cells, stably expressing HERG in a pcDNA3.1 vector, were selected with G418 (A.G. Scientific, San Diego, CA, USA;
Chapman et al. 2005). The cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 0.2 mg/ml G418. The human ML-1 follicular thyroid cancer cells were provided by Dr Johann Schönberger (University of Regensburg, Germany). The cells were cultured in DMEM supplemented with 10% FBS, 1% l-glutamine, and 1% penicillin/streptomycin. FTC-133 thyroid follicular cancer cells were obtained from Banca Biologica e Cell Factory, National Institute of Cancer Research (Genova, Italy). The cells were grown in DMEM and F-12 (Ham’s) medium (1:1) supplemented with 10% FBS and 2 mM l-glutamine. All cells were cultured in a humidified incubator at 37 °C and 5% CO 2. Cells were lipid-starved with medium containing 5% charcoal/dextran-treated FBS (lipid-stripped FBS) and serum starved with medium containing 0.2% FAF-BSA (serum-free medium (SFM)). We did not use any normal human thyroid cells for control purpose in this study, as S1P is without any effects on either proliferation or migration in normal thyroid cells (Balthasar et al. 2006).

Reverse transcriptase PCR (RT-PCR)
Total RNA was extracted from cells with Aurum Total RNA Mini Kit (Bio-Rad Laboratories) according to the manufacturer’s instructions. RNA quality and integrity was checked by absorbance spectroscopy and gel electrophoresis. cDNA was generated by reverse transcription reactions on 0.5 μg RNA using SuperScript III (Invitrogen) and oligo (dT) primers following the manufacturer’s instructions. Reaction mixtures without the reverse transcriptase enzyme were used as negative controls and to ensure that RNA preparations were not contaminated with genomic DNA. The primers for S1P1 were (forward) 5’-AAGGCTCAGTGTT-CATCGT-3’ and (reverse) 5’-GCTATTGTTGCTG-CTGCTTG-3’, yielding a 201 bp product. The thermo cycling settings were one cycle of denaturing phase of 5 min at 92–93 °C, 40 cycles of 30 s at 92–93 °C, 1 min annealing phase at 61 °C, and 1 min extension phase at 72 °C, with a final extension phase of 5 min at 72 °C. The MgCl₂ concentration was kept 2 mM. And the primers for S1P3 were (forward) 5’-AAGGCTCAGTGTT-CATCGT-3’ and (reverse) 5’-GCTATTGTTGCTG-CTGCTTG-3’, yielding a 201 bp product. The thermo cycling settings were one cycle of denaturing phase of 5 min at 90 °C, 40 cycles of 30 s at 90 °C, 1 min annealing phase at 61 °C, and 1 min extension phase at 72 °C, with a final extension phase of 5 min at 72 °C. The MgCl₂ concentration was kept 2 mM. The primers for

Cell migration assay
Migration assays were performed on 6.5 mm diameter Transwell inserts with 8 μm pore size. The membranes were coated with 5 μg/cm² collagen I for HEK-293 HERG cells and with human collagen IV for all other cell lines and allowed to dry overnight. The next day, the chambers were reconstituted for 1 h before the experiment with SFM at 37 °C. Cells were grown in SFM for 1 day before the experiment. Cells were added to the upper chamber in 200 μl SFM; 100 000 cells per insert were used with HEK-HERG cells and 50 000 cells per insert with other cell lines. Medium containing 5% lipid-stripped FBS was used as a chemoattractant and added to the lower chamber with or without 100 nM S1P in volume of 800 μl. In some experiments, the cells were pre-incubated with 10 μM JTE for 1 h, 1 μM VPC 23019 for 1 h; 10 μM Y-27632 for 1 h; 100 ng/ml C3 transferase for 4 h; 10 μM E-4031 for 1 h, 50 μM Rac1 for 30 min, 5 μM SK inhibitor for 15 min, or 3 μM PTEN inhibitor [bpV(phen)] for 1 h at 37 °C. The inhibitors were present in both the upper and the lower chambers. The cells were allowed to migrate for 6 h and the nonmigrated cells on top of the membrane were wiped off with a cotton swab. The migrated cells were fixed in 2% paraformaldehyde for 10 min and stained with 0.1% crystal violet in 20% methanol for 5 min. The membranes were rinsed in PBS and water and allowed to dry overnight. The cells were counted at 40× magnification in eight microscopic fields in a straight line bisecting the membrane.
Proliferation assay

Cell proliferation was measured with the $^3$H-thymidine incorporation method; 75 000 cells per 1.5 ml medium were plated in triplicate on 35 mm plates, grown for 24 h, and then lipid-starved for 24 h. Next, the cells were treated with S1P for 24 h. For the final 4 h, 0.4 µCi $^3$H-thymidine per ml of medium was added on the plates. After 4 h of incubation, the cells were washed three times with ice-cold PBS and incubated with 5% perchloric acid for 10 min, followed by incubation with 0.1 M NaOH for 10 min. Samples were transferred to scintillation vials and radioactivity was measured using a Wallac 1411 liquid scintillation counter.

Immunocytochemistry staining of C643 cells with HERG antibody. C643 cells (100 000) were grown on poly-L-lysine-coated glass coverslips in 35 mm plates for 24 h. Next day, the cells were serum-starved for 24 h. The cells were then stimulated with 100 nM S1P for 0, 30, and 60 min. The cells were washed three times with PBS and fixed with methanol–acetic acid (95:5) for 5 min at −70°C. After fixation, the cells were washed three times with PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 min. Cells were washed three times with PBS and blocked with 5% normal goat serum in PBS for 60 min. Cells were incubated with the anti-HERG antibody (1:100) overnight at +4°C. Next day, after three washes with ice-cold PBS, the cells were reblocked with 5% normal goat serum in PBS for 60 min and treated with the secondary antibody (Alexa Flour-568 goat anti-rabbit antibody, 1:500) for 1 h. In negative controls, the cells were only incubated with the secondary antibody. The slides were mounted with Mowiol mounting medium containing DAPI (1.5 µg/ml). Confocal images were obtained using the Zeiss LSM 780 laser scanning confocal microscope (Jena, Germany). Images were taken using 63× 1.2 water objective. The images were acquired with ZEN 2010 software.

Rac activation assays

Cells (50 000) were plated on 35 mm plates, grown until they reached 50% confluency, and serum-starved for 24 h. The cells were treated with S1P 100 nM for 0, 1, 3, 6, 12, and 30 min and lysates were made, aliquoted for protein concentration measurement, and snap-frozen immediately after harvesting and clarification as directed in user manual. The protein concentration was measured using the BSA kit. The protein concentration used for assays was kept at 0.5 mg/ml by equalization of protein concentration in each sample. The Rac activation assay was performed according to the manufacturer’s instructions.

Western blotting

C643 and HEK-293 HERG cells were grown in SFM for 24 h before treatment and stimulated with 100 nM S1P for 0, 30, 60, and 120 min. The protocol used for making whole cell lysates and for the western blotting has been described elsewhere (Balthasar et al. 2006). Densitometric analysis was performed using the Image J program for image analysis (NIH, Bethesda, MD, USA) and the results were corrected for protein loading by normalization with Hsc70 expression.

Transfection of C643 cells with siRNA using electroporation

Transfection of cells with siRNA was done by electroporation. Cells were detached in DMEM without additives and counted. Four million cells were pelleted and suspended in 200 µl OptiMem. siRNA (2 µM) was suspended in 200 µl OptiMem. The two suspensions were mixed and incubated for 15 min at room temperature. After the incubation, the suspension was pipetted into an electroporation cuvette. The cells were electroporated at 240 V and 500 µF. The electroporated cells were transferred to 100 mm cell culture plates to grow and after 48 h the cells were used to start the migration assay. Transfecting the cells with a control scrambled siRNA was without any effects on migration, compared with untransfected control cells.

Patch clamp studies

For electrophysiology, C643 cells and HEK-HERG cells grown in DMEM were plated at low density onto round glass coverslips kept in 24-well plates. HEK-HERG cells were grown under constant selection using 0.25 µg/ml G418. To obtain nominally lipid-free conditions, cells were exposed for 1–2 days to a SFM containing 0.2% FAF-BSA. The S1P (250 nM) experiments done with HEK-HERG cells were grown overnight in SFM.

The existence of HERG currents on C643 cells was probed using extracellular solutions (ECS) containing 150 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl$_2$, 1.2 mM MgCl$_2$, 5 mM HEPES, pH 7.4, and 100 mM NaCl, 55.5 mM KCl, 1.8 mM CaCl$_2$, 1.2 mM MgCl$_2$, and 5 mM HEPES, pH 7.4. The intracellular solution (ICS) contained 135 mM KGluc, 5 mM MgATP, 5 mM BAPTA, 2 mM MgCl$_2$, and 10 mM HEPES, pH 7.2. In HEK-HERG recordings, the ECS comprised 150 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl$_2$, 1.2 mM
MgCl₂, and 5 mM HEPES, pH 7.4 (supplemented with 1 mg/ml FAF-BSA), and the ICS contained 135 mM KCl, 5 mM MgATP, 5 mM BAPTA, 2 mM MgCl₂, and 10 mM HEPES, pH 7.2.

Whole-cell currents (Hamill et al. 1981) were recorded at 22–24 °C using an EPC-9 amplifier and Pulse software (HEKA Elektronik, Lambrecht, Germany). Series resistance was at least 80% compensated for and currents were sampled at 5–10 kHz and filtered at 1–2 kHz. Pipettes pulled from a thin-walled borosilicate glass (Harvard Apparatus Ltd, Edenbridge, UK) had a resistance of 3–6 MΩ when filled with above-mentioned ICSs. The voltage-pulse protocols used was constituted from an 800 ms step to +40 mV that was followed by a series of 200 ms rectangular pulses from +10 to −130 mV at 10 mV decrements and was used to construct the IV profile of HERG tail currents. Cells were held at −70 mV between pulses. The above-mentioned voltage values were corrected off-line for a liquid junction potential (LJP) of 17 mV in C643 experiments and for a LJP of 5 mV in HEK-HERG experiments. LJP values were estimated with the JPCalc software (Barry 1994).

Data analysis and illustrations were done with PulseFit (HEKA Elektronik) and Origin (OriginLab, Northampton, MA, USA) software. Leak current estimated with a P/4 protocol at −70 mV was subtracted after which the peak tail currents were detected.

### Statistical analysis

Results are presented as mean ± S.E.M. for at least three independent measurements. The data were normalized to minimize the variations within the groups and between groups by normalizing against control mean to make data comparable to different experiments. The curve fitting and analysis of statistical significances was performed by one-way ANOVA and Bonferroni’s post hoc test for selected samples, using the GraphPad Prism 4 program (GraphPad Software Inc., San Diego, CA, USA). The statistical significance of patch-clamp data was tested with nonpaired Student’s t-test. A P value <0.05 was considered statistically significant.

### Results

S1P receptor expression in ATC cell lines

S1P receptors are well known for their regulatory role in cell migration and proliferation. Previously, we investigated the effect of S1P on both proliferation and migration of several thyroid cancer cell lines and

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**Figure 1** Expression of S1P1–3 in C643 and THJ-16T cells. (A) Western blot showing the expression of S1P1–3 in C643 and THJ-16T cells. ML-1 is used as positive control. Hsc70 is used as loading control. (B) RT-PCR on RNA from C643, THJ-16T, and ML-1 cells. ML-1 cells are used as positive control. — denotes negative control (without reverse transcriptase). The product lengths were S1P₁, 223 bp; S1P₂, 197 bp, and S1P₃, 201 bp. hHPRT was used as a loading control. The blots are representative of at least three separate experiments.
normal thyroid cells (Balthasar et al. 2006). However, the origin of some of the cell lines later proved to be unclear (Schweppe et al. 2008). Thus, we investigated S1P receptor expression and function in verified human anaplastic C643 and THJ-16T thyroid cancer cell lines (Schweppe et al. 2008, Marlow et al. 2010). RT-PCR and western blotting results showed that S1P1, S1P2, and S1P3 are expressed at both mRNA and protein levels in these cell lines (Fig. 1). Interestingly, the receptor profile is similar to that observed in the follicular thyroid cancer cell lines ML-1 and FTC-133 (Balthasar et al. 2006, Bergelin et al. 2009).

Effects of S1P on migration and proliferation of C643 and THJ-16T cells

In follicular ML-1 thyroid cancer cells, S1P has a stimulatory effect on migration (Balthasar et al. 2006). However, both in C643 and THJ-16T cells, and in the follicular FTC-133 cancer cells, S1P inhibited migration (Fig. 2A). The effect was concentration dependent (results not shown). We continued to characterize by which mechanism S1P attenuated migration using C643 cells. As can be seen in Fig. 2B and C, the inhibitory effect of S1P was blocked by the S1P2 antagonist JTE-013, but not by the S1P1,3 antagonist VPC23019. Furthermore, knockdown of S1P2 by siRNA abolished the effect of S1P on C643 cell migration (Fig. 2D). Thus, the results show that the effect of S1P was mediated by S1P2. We also investigated the effect of S1P on the proliferation of C643, THJ-16T, and FTC-133 cells. Our results show that the concentration of S1P that strongly attenuated cell migration was without an effect on the proliferation of these cell lines (Fig. 3).

Previous investigations have shown that S1P2 inhibits migration through Rho and ROCK pathways (Okamoto et al. 2000, Lepley et al. 2005, Takashima et al. 2008). Pre-incubation of the cells with either the Rho inhibitor C3-transferase (100 ng/ml for 4 h) or the ROCK inhibitor Y-27632 (10 μM for 1 h) slightly, but significantly, enhanced basal migration of the cells (Fig. 4A and B). In cells treated with C3-transferase

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**Figure 2** S1P inhibits migration of thyroid cancer cells lines by activating S1P2. (A) Effect of S1P on migration induced by 5% lipid-stripped FBS in C643, THJ-16T, and FTC-133 cells. The number of migrated cells in controls was 532.8 ± 32.2 for C643 cells, 372.0 ± 14.1 for THJ-16T cells, and 263.3 ± 19.6 for FTC-133 cells (mean ± s.d.). (B) The S1P2 inhibitor JTE-013 attenuates the effect of S1P on migration of C643 cells. (C) Lack of an effect of the S1P1,3 inhibitor VPC23019 on the S1P-evoked attenuation of migration of C643 cells. C siRNA denotes cells treated with a control scrambled siRNA. The normalized results in the graphs are the mean ± s.e.m., n = 3. Asterisks (*) indicate statistically significant differences in migration compared with control. Data were analyzed with one-way ANOVA and Bonferroni’s post hoc test (**P < 0.01; ***P < 0.001).
and Y-27632, the S1P-evoked decrease in migration was abolished (Fig. 4A and B). In contrast to Rho, Rac promotes cell migration and a more invasive phenotype (Keely et al. 1997). Pre-incubation with a Raci (50 μM for 30 min) clearly attenuated the migration of C643 cells. In addition, stimulating cells treated with the Raci with S1P further decreased the migration (Fig. 4C). Furthermore, using a Rac activation assay, we could show that S1P significantly attenuated Rac activity (Fig. 4D).

PTEN and sphingosine kinase as modulator of migration

Investigations have shown that S1P2 may also activate PTEN and through inhibition of Akt attenuate migration (Sanchez et al. 2005). In C643 cells pre-incubated with 3 μM of the PTEN inhibitor [bpV(phen)] for 1 h, the effect of S1P on migration was significantly attenuated but not abolished (Fig. 5A). [bpV(phen)] had no effect on basal migration.

We have previously shown with ML-1 cells that sphingosine kinase enhances ML-1 cell migration through an autocrine mechanism (Bergelin et al. 2009). Pretreatment of C643 cells with a SKi (5 μM for 15 min) potently enhanced basal migration of the cells (Fig. 5B). In cells pretreated with SKi, S1P attenuated migration. The results suggest that sphingosine kinase may, in fact, also function as an inhibitor of migration.

HERG potassium channels as modulators of migration and proliferation

Several ion channels, including the hKv 11.1 (HERG) potassium channel, are important modulators of cancer cell migration and proliferation (Arcangeli 2005, Prevarskaya et al. 2010). In C643 cells, the HERG channel protein could be detected both at mRNA and protein level (Fig. 6A and B). Furthermore, HERG was detected in commercial samples of normal human thyroid mRNA (Fig. 6B). Due to a rapid inactivation, HERG tail currents show marked inward rectification (Sanguinetti et al. 1995, Smith et al. 1996). In patch-clamp studies, we were unable to detect currents resembling HERG tail currents in C643 cells (Fig. 6F). However, the membrane of C643 cells did show some K+ permeability, as the increase in extracellular potassium concentration ([K+]o) from 5.4 to 55.55 mM shifted the IV curve to a depolarizing direction (Fig. 6F, inset), resulting in a change in the reversal potential of membrane currents (E<sub>R</sub>) from −46.0 ± 1.3 to −23.5 ± 0.9 mV (n = 12–13, P < 0.01). The above results thus indicate that the cells express HERG protein, although the channel apparently is not conducting any current.

Several investigations have indicated that also nonconducting HERG proteins modulate cell function (Kaczmarek 2006). We observed that pretreatment of the cells with the HERG inhibitor E-4031 (10 μM for 1 h) significantly decreased migration. Previous investigations have suggested that activation of Rho may attenuate HERG function (Storey et al. 2002). As the inhibitory effect of S1P on migration apparently is mediated by Rho, we investigated whether S1P could modulate the effect of E-4031 and the HERG activator PD 118057. Our results showed that S1P significantly enhanced the effect of E-4031 (Fig. 6C). PD 118057 (10 μM) evoked a slight, but significant, migration (Fig. 6D). In these cells, S1P attenuated migration (Fig. 6C and D).

To further characterize the importance of HERG in regulating migration of C643 cells, the cells were transfected with HERG siRNA. Interestingly, our results showed that the basal migration was slightly decreased in HERG downregulated cells, compared with control cells, and that the inhibitory effect of E-4031 was abolished. However, in these cells, S1P still decreased migration (Fig. 6E).
As receptor-mediated signaling has previously been shown to decrease HERG expression and function (Ramstrom et al. 2010), we next investigated whether S1P could affect the expression of HERG in C643 cells. Using both immunochemistry and western blot experiments, we could detect a transient decrease in HERG expression in C643 cells after 30 min of stimulation with 100 nM with S1P (Fig. 7A, B, and C).

Effects of S1P on the migration of HEK-HERG cells

We have previously shown that HERG is an important regulator of the migration and proliferation of HEK cells overexpressing HERG (Afrasiabi et al. 2010). As S1P seems to regulate HERG expression, we used HEK-HERG cells as a model system for further experiments. Treatment of HEK-HERG cells with 100 nM S1P decreased the expression of HERG protein (Fig. 8 A and B). The HEK-HERG cells exhibited robust tail currents under 5.4 mM [K\(_{\text{C}}\)] conditions (Fig. 8C). Preliminary experiments revealed that a prolonged incubation of HEK-HERG cells under SFM conditions suppressed tail currents markedly. The relative amplitude of 100 \(\mu\)M (\(n=23\)) measured at \(-135\) mV was decreased after 1 day to \(58 \pm 4\%\) of control (\(n=35, P<0.05\)) and after 2 days to \(36 \pm 3\%\) of control (\(n=17, P<0.05\), data not shown) in SFM. Experiments with S1P were preceded by a 1-day pre-incubation in SFM. The HERG IV profile (Fig. 8C, inset) showed that treatment of the cells with 100 nM S1P for 1–2 h reduced the tail currents at all voltages below \(-90\) mV (\(n=20–35, P<0.01\)). This clearly shows that also HERG proteins forming functional channels are modulated by S1P. In addition, 100 nM S1P also decreased the migration of the cells (Fig. 8D).

Discussion

In the present report, we show for the first time that the S1P receptors S1P\(_{1,2,3}\) are expressed in human ATC C643 and THJ-16T cells at both mRNA and protein levels. Previously, we had reported the expression profile of S1P receptors on mRNA and protein levels in normal human thyroid cells and in different thyroid cancer cell lines (Balthasar et al. 2006). However, the origin of some of the cell lines we used was later proven to be unclear (Schweppe et al. 2008). To investigate whether S1P may affect ATC cells, we investigated the ATC cell lines C643 and THJ-16T, which have recently been proven to be original cancer cells (Schweppe et al. 2008, Marlow et al. 2010). Our results showed that physiological concentrations of
S1P (200–900 nM in blood plasma and 400–1100 nM in serum; see Takabe et al. (2008)) inhibited the migration of both C643 and THJ-16T cells. S1P also inhibited the migration of the follicular thyroid cancer FTC-133 cells, and HEK-293 cells stably overexpressing the HERG potassium channel. Interestingly, all the above-mentioned cell lines have the same receptor profile as follicular thyroid ML-1 cancer cells. In the ML-1 cells, however, S1P potently enhanced migration at low nanomolar concentrations (Balthasar et al. 2006). This is an important observation and clearly indicates that the S1P receptor profile per se cannot be used as a marker for a migratory phenotype of thyroid cancer. Whether this also holds true for papillary thyroid cancer cells is still an open question but is subject of a further study.

We characterize the mechanism and showed that the inhibitory effect of S1P on C643 cell migration is mediated by S1P2. We base our conclusion on the following observations: the S1P2 antagonist JTE-013, and S1P2 siRNA, attenuated the effect of S1P, whereas the S1P1,3 antagonist VPC23019 was without any effects. Furthermore, S1P2 couples to G12/13 and activates the Rho small GTPase (Lepley et al. 2005). Activation of Rho inhibits Rac, an important regulator of cell migration. In line with previous reports (Gonda et al. 1999, Windh et al. 1999, Okamoto et al. 2000, Ishii et al. 2004), we show that inhibition of Rho with C3 transferase abolished the effect of S1P. In addition, by inhibiting Rac pharmacologically, migration of C643 cells was attenuated, and Rac activity was transiently inhibited by S1P. Furthermore, the ROCK inhibitor Y-27632 attenuated (but did not inhibit) the effect of S1P. S1P did not completely abolish migration of C643 cells.

In many different cancers, SphK1 is overexpressed and seems to correlate with a more severe clinical phenotype (Gault & Obeid 2011). We have previously shown that overexpression of SphK1 in ML-1 thyroid cancer cells enhanced migration of these cells but that in FTC-133 cells, SphK1 overexpression decreased migration (Bergelin et al. 2009). Furthermore, in a recent study, SphK1 was shown to be overexpressed in several cell lines, promoting proliferation of these cells (Guan et al. 2011). In addition, Guan et al. (2011) showed that several thyroid cancers are overexpressing SphK1 and that the overexpression correlates with the degree of malignancy. As the migration of FTC-133 cells was decreased by S1P, we blocked SphK1 in the C643 cells using the inhibitor SKi. In these cells, the basal migration was significantly increased but was attenuated when exogenous S1P was readded. S1P produced by SphK1 may be transported out from the cells and activate S1P receptors through an autocrine effect described previously (Bergelin et al. 2009). Our results thus indicate that SphK may, in some instances, evoke an antimigratory effect. We conclude that the receptor profile of the cells, again, may decide the outcome of such an autocrine S1P signaling.

We were not able to observe any significant effects of S1P on the proliferation of C643, THJ-16T, or FTC-133 cells. Interestingly, in ML-1 cells, S1P slightly but significantly decreased proliferation (Balthasar et al. 2006). In addition, in ML-1 cells overexpressing SK,
proliferation was slightly decreased (Bergelin et al. 2009). Presently, we do not have any explanation for why S1P does not affect proliferation in either C643, THJ-16T, or FTC-133 cells. However, in primary thyroid cell cultures, S1P does not affect proliferation of the cells (Balthasar et al. 2006).

**HERG** potassium channels and HERG-related currents are often encountered in various cancer cells (Arcangeli et al. 1995, Bianchi et al. 1998, Prevarskaya et al. 2010), but marked protein expression is not always paralleled by HERG like currents. However, HERG antagonists reduced proliferation and migration.
Figure 7 S1P-evoked downregulation of HERG protein expression in C643 cells. (A) Western blot showing a time-dependent effect of S1P (100 nM) on HERG protein expression. Hsc70 is used as loading control. (B) Summary of three separate experiments as shown in A. The results are the mean ± S.E.M., n=3; * indicates statistically significant differences in migration compared to control. Data were analyzed with one-way ANOVA and Bonferroni’s post hoc test (*P<0.05). (C) Confocal images of C643 cells expressing HERG after stimulation with S1P. a (DAPI), b (HERG), and c (Merge): C643 cells not stimulated with S1P. d (DAPI), e (HERG), and f (Merge): C643 cells stimulated with 100 nM S1P for 30 min. g (DAPI), h (HERG), and i (Merge): C643 cells stimulated with 100 nM S1P for 60 min. j, DAPI-stained C643 cells incubated with secondary antibody only. The images shown are representative images of three separate experiments. The scale bar is 20 μm.
also in the absence of currents (Hegle et al. 2006, Afrasiabi et al. 2010), and overexpression of both conductive and nonconductive variants of *Drosophila* EAG promoted cell growth (Hegle et al. 2006). Thus, transmembrane ion fluxes may not be necessary for the proliferative or migratory effects of HERG channels. Also other voltage-gated ion channels may have functions that extend beyond ion conduction (Kaczmarek 2006). The C643 cancer cells expressed HERG potassium channels both at mRNA and protein levels. Our results showed that in these cells, the HERG inhibitor E-4031 slightly attenuated migration. The effect of E-4031 was enhanced by S1P. In ML-1 cells, blocking HERG also attenuated migration (N Bergelin and K Törnquist, unpublished observations 2010). Furthermore, stimulating C643 cells with S1P decreased HERG protein content, as shown using both western blot and immunohistochemistry.

In control experiments, we used HEK cells stably overexpressing HERG (HEK-HERG). We have previously shown that inhibition of HERG attenuated migration in these cells (Afrasiabi et al. 2010). We now show that S1P attenuated migration of HEK-HERG cells and that S1P in these cells evoked a decrease in HERG protein, similar to that seen in C643 cells. This effect probably is the result of S1P receptor-evoked production of diacylglycerol and an internalization and possible degradation of the channel previously seen in HEK-HERG cells stimulated with carbachol (Ramstrom et al. 2010). There was, however, a difference in the time course of the S1P-evoked HERG protein decrease between C643 cells and HEK-HERG cells. The reason for this is not known but could possibly relate to the fact that the HERG protein was substantially overexpressed in HEK-HERG cells.

In conclusion, we have shown that S1P can attenuate migration of several thyroid cell lines. The receptor profile of the cancer cells seems to be an important factor when deciding the outcome of the stimulation but cannot be used as a prognostic factor regarding the migratory potential of the cells. Furthermore,

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**Figure 8** Effect of S1P on HERG protein expression and current and on migration of HEK-HERG cells. (A) Western blot showing a time-dependent effect of S1P (100 nM) on HERG protein expression in HEK cells stably overexpressing HERG (HEK-HERG cells). Hsc70 is used as loading control. (B) Summary of three separate experiments as shown in A. The results are the mean ± S.E.M., n = 3. Data were analyzed with one-way ANOVA and Bonferroni’s post hoc test (*P < 0.05). (C) The presence of massive tail currents in HEK-HERG cells. The inset describes the inward rectifying IV profile of HERG currents before (open circles, n = 35) and after a S1P incubation of 1–2 h (crossed circles, n = 20). Five traces of a full voltage step protocol are shown and the tail current segment is indicated with a horizontal line. Statistical significance was tested using Student’s *t*-test (*P < 0.01). (D) S1P attenuates the migration of HEK-HERG cells. The results are the mean ± S.E.M., n = 3. Data were analyzed with one-way ANOVA and Bonferroni’s post hoc test (***P < 0.001).
HERG potassium channel protein is, at least in part, participating in the regulation of the migratory response.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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