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Sphingosine 1-phosphate receptors modulate intracellular Ca^{2+} homeostasis

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Abstract

Ligation of sphingosine 1-phosphate (S1P) to a set of specific receptors named S1P receptors (S1PRs) regulates important biological processes. Although the ability of S1P to increase cytosolic Ca^{2+} in various cell types is well known, the role of the individual S1PRs has not been fully characterized. Here, we provide a complete analysis of S1P-dependent intracellular Ca^{2+} homeostasis in HeLa cells. Overexpression of $S1P_2$, or $S1P_3$, but not $S1P_1$, leads to a significant increase in cytosolic and mitochondrial $[Ca^{2+}]$ in response to S1P challenge. Moreover, cells ectopically expressing $S1P_2$, or $S1P_3$ exhibited an appreciable decrease of the free Ca^{2+} concentration in the endoplasmic reticulum, dependent on stimulation of receptors by S1P endogenously present in the culture medium which was accompanied by a reduced susceptibility to C_2-ceramide-induced cell death. These results demonstrate a differential contribution of individual S1PRs to Ca^{2+} homeostasis and its possible implication in the regulation of cell survival.

Keywords: Sphingosine 1-phosphate; Calcium; Apoptosis; Ceramide; Sphingosine 1-phosphate receptors

Within the last decade the importance of some sphingolipid metabolites, such as ceramide and sphingosine 1-phosphate (S1P), as regulators of many cellular functions has become clear, and have lead to an explosion of interest for these molecules [1]. It is now widely accepted that most if not all the effects of S1P are exerted from outside the cells via ligation to G-protein-coupled receptors, which have been named S1P receptors (S1PRs). To date, five closely related S1PRs ($S1P_1$–$S1P_5$) have been identified [2]. S1P appears to be able to modulate the intracellular Ca^{2+} signaling through the action of $S1P_2$ and $S1P_3$, which mobilize the cation from intracellular stores (namely endoplasmic reticulum (ER)) [3,4], while the ability of $S1P_1$ to mobilize intracellular Ca^{2+} is still matter of debate. In native C2C12 cells Ca^{2+} mobilization induced by S1P was found to be a receptor-mediated process that involves $S1P_2$ and $S1P_3$, but not $S1P_1$ [5]. Since Ca^{2+} is considered one of the most important intracellular messengers, Ca^{2+} signals need to be flexible and precisely regulated. In this process, mitochondria and ER have a pivotal role because they are not only involved as modulators of the variations in Ca^{2+} concentration, but also as Ca^{2+} intracellular targets. Although it has been extensively demonstrated that S1P is able to induce an increase in cytosolic Ca^{2+} concentration in various cell types [2,6], the kinetics of Ca^{2+} signaling in mitochondria and ER, and the role of the different S1PRs in modulating Ca^{2+} homeostasis in these organelles, have yet to be investigated.

Abbreviations: S1P, sphingosine 1-phosphate; ER, endoplasmic reticulum; S1PRs, sphingosine 1-phosphate receptors; $[Ca^{2+}]_{cyt}$, cytosolic Ca^{2+} concentration; $[Ca^{2+}]_m$, mitochondrial Ca^{2+} concentration; $[Ca^{2+}]_{er}$, endoplasmic reticulum Ca^{2+} concentration; GFP, green fluorescent protein; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; BSA, bovine serum albumin; PTx, pertussis toxin; KRB, Krebs–Ringer modified buffer; SDS, sodium dodecylsulfate.

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Using recombinant aequorin probes targeted to subcellular organelles we here provide a complete analysis of intracellular Ca^{2+} homeostasis. In this study, we demonstrate that HeLa cells overexpressing $S1P_2$ or $S1P_3$, but not $S1P_1$ receptor showed a transient increase in cytosolic and mitochondrial Ca^{2+} concentration upon challenge with S1P.

Notably, in HeLa cells overexpressing $S1P_2$ or $S1P_3$, free Ca^{2+} concentration in the ER at the steady state was found to be decreased and intriguingly, both cell populations resulted to be less sensitive to C_2-ceramide-induced apoptosis, supporting a role for these receptors in the anti-apoptotic action of S1P.

Materials and methods

Materials. Coelenterazine was purchased from Molecular Probes, D-erythro-sphingosine 1-phosphate was from Calbiochem, pertussis toxin (PTx) from Alexis Corporation. All the other reagents were from Sigma–Aldrich. pcDNA3 vectors encoding for $S1P_1$, $S1P_2$, and $S1P_3$ were a kind gift of Prof. Y. Igarashi, Hokkaido University, Sapporo, Japan.

Cell culture and transfection. HeLa cells were grown in DMEM, supplemented with 10% FCS. For aequorin measurements, cells were co-transfected for 36 h with 3 μg of cDNA encoding for $S1P_1$, or $S1P_2$, or $S1P_3$ or the empty vector, and 1 μg of cDNA for cytosolic aequorin [7] (pcDNA1-cytAEQ) or 1 μg of cDNA for mitochondrial aequorin [8] (VR1012-mtAEQmut) or 1 μg of cDNA for aequorin localized in the ER [9] (VR1012-erAEQ). For microscopic analysis of GFP-expressing cells, cells were transfected with 8 μg of DNA (2 μg VR1012-erGFP or pEGFP together with 6 μg pcDNA3 or pcDNA3-$S1P_1$, or pcDNA3-$S1P_2$, or pcDNA3-$S1P_3$). In all cases, transfection was carried out using the Ca^{2+}-phosphate technique.

RT-PCR. One microgram of total RNA extracted from HeLa cells transfected with pcDNA3-$S1P_1$, or pcDNA3-$S1P_2$, or pcDNA3-$S1P_3$, or pcDNA3, was reverse transcribed into DNA and subjected to PCR using the following software-designed oligonucleotide primers: $S1P_1$ 5′-CCT ATCATGGGCTGGAACTGCATC-3′ (sense), 5′-GGGTTGTCC CCAT CGTCCTTCTGGGG-3′ (antisense); $S1P_2$ 5′-TGCCGCATGCTTCTG CTCATCGGG-3′ (sense), 5′-TCAGACCACCGTGTTGCCCTCCAG-3′ (antisense); $S1P_3$ 5′-CTGCTCTACCATCCTGCCCCTCTAC-3′ (sense), 5′-GTTGCAGAAGATCCCATTCTGAAGTGC-3′ (antisense); β-actin 5′-GCGGGAAATCGTGCGTGACATT-3′ (sense), 5′-GATGGAGTT GAAGGTAGTTTCGTG-3′ (antisense), all synthesized by Pharmacia Biotech (Uppsala, Sweden). PCR amplification products were separated on a 1.2% agarose gel.

Aequorin measurements. Aequorin reconstitution, luminescence measurements, and calibration into $[Ca^{2+}]$ values were carried out as previously described [10]. For mtAEQ and cytAEQ, 36 h after transfection the coverslips with the cells were incubated with 5 μM coelenterazine for 1–2 h in KRB (Krebs–Ringer modified buffer: 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM Na_2HPO_4, 5.5 mM glucose, 20 mM $NaHCO_3$, 2 mM L-glutamine, and 20 mM Hepes, pH 7.4 at 37 °C), and then transferred to the perfusion chamber. In order to reconstitute with high efficiency the erAEQ, the luminal $[Ca^{2+}]$ of this compartment was at first reduced by incubating the cells for 1 h at 4 °C in KRB supplemented with 5 μM coelenterazine, the Ca^{2+} ionophore ionomycin and 600 μM EGTA. The cells were then extensively washed with KRB supplemented with 2% BSA before the luminescence measurement. All experiments were carried out in KRB, supplemented with 1 mM $CaCl_2$. Agonists were added to the same medium.

Microscopic analysis. HeLa cells were co-transfected with VR1012-erGFP plus pcDNA3 or pcDNA3-$S1P_1$, or pcDNA3-$S1P_2$, or pcDNA3-$S1P_3$, 36 h after transfection were loaded with mitoTracker Red, 25 nM in KRB, to visualize mitochondria. Using an inverted Nikon microscope fluorescence images were captured by a back-illuminated CCD camera using the Metamorph software.

Cell survival measurement. HeLa cells were co-transfected with empty vector of S1PR-encoding plasmids together with erGFP-encoding vector for 36 h and then incubated in DMEM without serum in the presence of 10 μM C_2-ceramide overnight or 1 μM staurosporine for 4 h. Apoptotic response was evaluated by counting in at least 50 microscopic fields fluorescent surviving cells.

Statistical analysis. The data were analyzed by Student's *t*-test and $p < 0.05$ was considered significant. Densitometric analysis of the bands was performed using Imaging and Analysis Software by Bio-Rad (Quantity-One).

Results

Effect of S1PR overexpression on cytosolic, mitochondrial Ca^{2+} responses, and ER Ca^{2+} concentration

In a first series of experiments, by RT-PCR analysis we found that $S1P_1$, $S1P_2$, and $S1P_3$ were expressed, at least at mRNA level, in native HeLa cells as expected, cells transfected with plasmids encoding for $S1P_1$, or $S1P_2$, or $S1P_3$, exhibited mRNA levels higher in comparison with control cells (Fig. 1A). Considering an average transfection efficiency of 30%, it can be estimated that in transfected cells recombinant mRNA of S1PRs was expressed 6 ± 1.5-fold (for $S1P_1$), 4 ± 0.1-fold (for $S1P_2$), and 10 ± 0.4-fold (for $S1P_3$) than the endogenous counterpart (Fig. 1A).

We then investigated the effect of individual S1PR on cytosolic and mitochondrial Ca^{2+} homeostasis using two specifically targeted chimeras of the Ca^{2+} sensitive photoprotein aequorin, the cytosolic (cytAEQ) and mitochondrial (mtAEQmut) aequorin. When controls were stimulated with 250 nM S1P no increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) could be observed. Similarly, $S1P_1$-overexpressing cells were unresponsive for S1P challenge. On the contrary, a small cytosolic Ca^{2+} peak was detected when $S1P_2$- or $S1P_3$-overexpressing cells were challenged with the sphingolipid (the Ca^{2+} increases were 0.78 ± 0.15 μM for $S1P_2$ and 0.72 ± 0.14 μM for $S1P_3$, $n = 11$) (Fig. 1B).

The variation in intracellular Ca^{2+} homeostasis, following histamine administration to HeLa cells has been previously well characterized [11,12], thus we challenged native and S1PR-overexpressing cells with 100 μM histamine. Unexpectedly, while native and $S1P_1$-overexpressing cells were showing an increase in $[Ca^{2+}]_{cyt}$ comparable to values previously reported in other studies (3.04 ± 0.42 μM, $n = 6$ and 2.75 ± 0.38 μM, $n = 9$, respectively), the Ca^{2+} peaks of $S1P_2$- or $S1P_3$-overexpressing cells were much lower (1.11 ± 0.2 μM, $n = 5$ and 2.55 ± 0.21 μM, $n = 5$ vs 3.04 ± 0.42 μM of controls $p < 0.001$ and $p < 0.05$, respectively) (Fig. 1C).

Then, we analyzed mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$) responses in HeLa cells. We obtained data very similar to those recorded in the cytosol. As shown in Fig. 1D, when native or $S1P_1$-overexpressing cells were challenged with 250 nM S1P, no $[Ca^{2+}]_m$ increase was monitored, whilst, when $S1P_2$- or $S1P_3$-overexpressing cells were treated with 250 nM S1P small mitochondrial Ca^{2+}

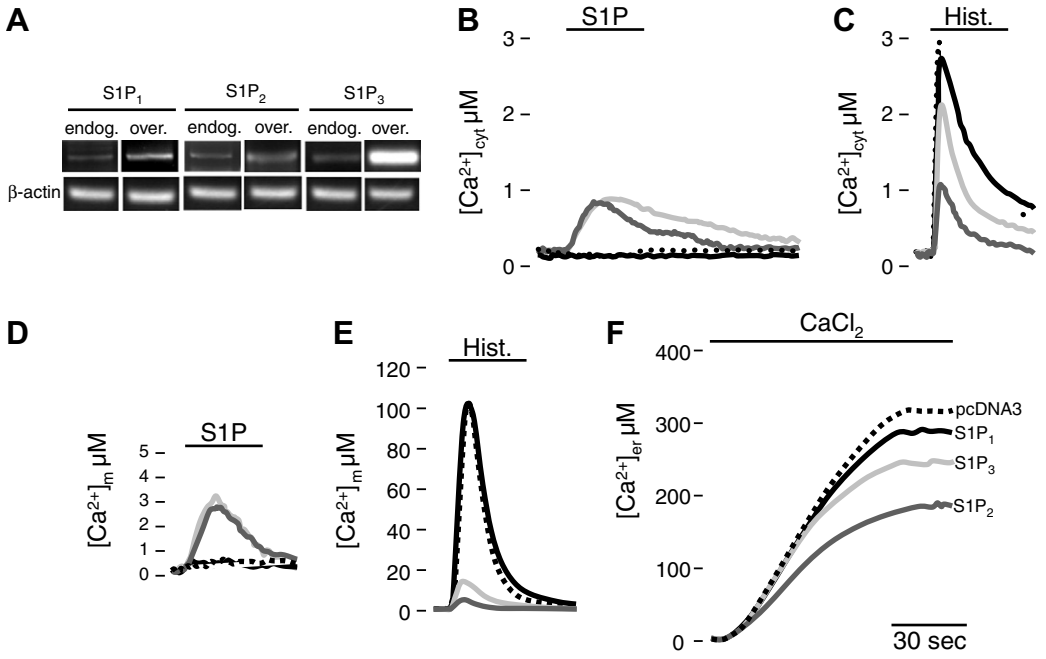

Fig. 1. Transcript levels of S1PR isoforms and effect of S1PR overexpression on cytosolic and mitochondrial and ER Ca^{2+} homeostasis in HeLa cells. (A) Native HeLa cells and cells transfected with pcDNA3-$S1P_1$, or pcDNA3-$S1P_2$, or pcDNA3-$S1P_3$ were subjected to RT-PCR analysis. β-Actin was used as an internal reference. $[Ca^{2+}]_{cyt}$ (B,C), $[Ca^{2+}]_m$ (D,E), and $[Ca^{2+}]_{er}$ (F) were measured in HeLa cells co-transfected with pcDNA3-$S1P_1$, or pcDNA3-$S1P_2$, or pcDNA3-$S1P_3$, or pcDNA3 alone, and pcDNA1-cytAEQ or VR1012-mtAEQmut or VR1012-erAEQ (black, gray, light gray, and dotted black traces, respectively). Where indicated, cells were treated with 250 nM S1P (B,D) or 100 μM histamine (C,E) added to the perfusion medium, or after Ca^{2+} depletion in the ER, stores were refilled with Ca^{2+} by perfusing the cells with KRB containing 1.2 mM $CaCl_2$, until a plateau was reached (F). Bands and traces are representative of >5 trials.

transients were recorded (3.0 ± 2.1 μM and 3.42 ± 2.38 μM, $n = 10$, respectively). Again, when stimulated with 100 μM histamine control or $S1P_1$-overexpressing cells were showing an increase in $[Ca^{2+}]_m$ of 84 ± 27 μM and 106 ± 37 μM, $n = 8$, respectively), while $[Ca^{2+}]_m$ peaks in $S1P_2$- or $S1P_3$-overexpressing cells were significantly lower (3.77 ± 2.12 μM, $n = 7$ and 13.1 ± 2.94 μM, $n = 7$, respectively, $p < 0.001$ vs control values) (Fig. 1E). We tested whether the decrease of histamine-induced cytosolic Ca^{2+} increase and mitochondrial Ca^{2+} uptake in cells overexpressing $S1P_2$ or $S1P_3$ could be explained by an impairment of the state of filling of the intracellular Ca^{2+} stores. For this purpose, the $[Ca^{2+}]$ of the ER lumen ($[Ca^{2+}]_{er}$) was measured with a specifically ER-targeted aequorin (erAEQ) chimera. The ER was first depleted of Ca^{2+}, cells were then transferred to the luminometer chamber, and store refilling was started by supplementing the perfusion medium with physiological $[Ca^{2+}]$. As illustrated in Fig. 1F, a significant difference in the $[Ca^{2+}]_{er}$ steady state could be appreciated between $S1P_2$- and $S1P_3$-overexpressing cells and control cells (191 ± 36 μM, $n = 10$ or 246 ± 58 μM, $n = 11$ vs 350 ± 39 μM, $n = 10$, $p < 0.05$ in both cases), indicating that overexpression of $S1P_2$ or $S1P_3$ reduces the state of filling of the Ca^{2+} stores. Conversely, the $[Ca^{2+}]_{er}$ steady state in $S1P_1$-overexpressing cells was not appreciably lower than controls (308 ± 52 μM, $n = 9$ vs 350 ± 39 μM, $n = 10$).

Effect of S1PR overexpression on organelle morphology and distribution

It was then investigated whether the alteration of ER Ca^{2+} homeostasis at the steady state brought about by the overexpression of $S1P_2$ or $S1P_3$ could be ascribed to an impairment of the morphology or distribution of intracellular organelles, such as mitochondria and ER. For this purpose, HeLa cells were co-transfected with a plasmid encoding for the green fluorescent protein (GFP) mutant targeted to the ER (VR1012-erGFP) and pcDNA3-$S1P_1$, or pcDNA3-$S1P_2$, or pcDNA3-$S1P_3$, or the empty vector. Cells then were loaded with mitoTracker, to visualize mitochondria, and analyzed by digital imaging fluorescence microscopy. The visualization of ER and mitochondria revealed the typical morphology and distribution of these two organelles, i.e., a homogeneous network throughout the cells for ER, and a rod-like morphology for mitochondria in all conditions (Fig. 2), ruling out that the observed decrease of free $[Ca^{2+}]_{er}$ in $S1P_2$- and $S1P_3$-overexpressing cells could be due to the alteration of morphology or distribution of intracellular organelles.

Effect of pertussis toxin on ER Ca^{2+} concentration

Since FCS present in the culture medium contains small amounts of S1P, we investigated whether the decrease in

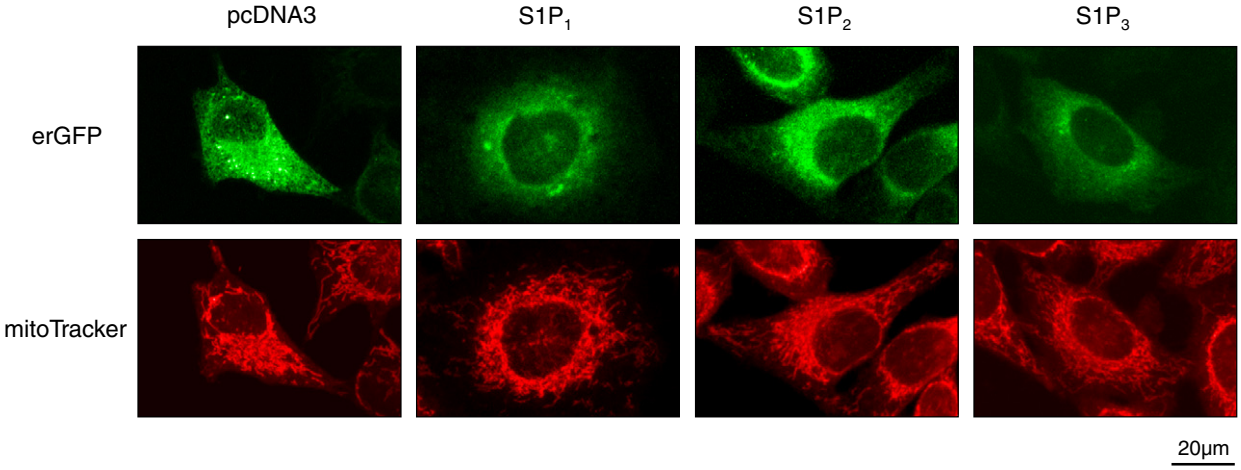

Fig. 2. Morphology and distribution of mitochondria and ER in HeLa cells overexpressing S1PRs. HeLa cells co-transfected with pcDNA3-$S1P_1$, or pcDNA3-$S1P_2$, or pcDNA3-$S1P_3$, or pcDNA3 alone, and VR1012-erGFP were loaded with mitoTracker Red to visualize mitochondria. Images are representative of >10 microscopic fields of three independent experiments.

$[Ca^{2+}]_{er}$ at the steady state was due to the occupancy of the ectopically expressed $S1P_2$ or $S1P_3$ receptors by the sphingolipid present in serum. For this purpose, we treated the different cell populations with 100 ng/ml of PTx, which disrupts G_i-mediated signaling, and is known to affect at various extent Ca^{2+} release induced by cell challenge with S1P [6]. As shown in Fig. 3, 16 h treatment with PTx of $S1P_2$- or $S1P_3$-overexpressing cells led to an increase in $[Ca^{2+}]_{er}$ at the steady state of approximately 18% and 27% (214 ± 8 μM, $n = 5$ vs 191 ± 36 μM, $n = 10$ and 314 ± 7, $n = 5$ vs 246 ± 58 μM, $n = 11$, $p < 0.05$ in both cases). PTx treatment of control or $S1P_1$-overexpressing HeLa cells did not alter the $[Ca^{2+}]_{er}$ (data not shown). These results show that in HeLa cells the $S1P_2$- and $S1P_3$-directed regulation of Ca^{2+} homeostasis involves, at least in part, G_i-dependent signaling events. Moreover, given that inhibition of $S1P_2$- or $S1P_3$-mediated G_i-coupled pathways lead to a partial recovery of $[Ca^{2+}]_{er}$ at the steady state, these data support the hypothesis that $S1P_2$ and $S1P_3$ were chronically activated by S1P endogenously present in FCS added to culture medium. In agreement, the decrease in $[Ca^{2+}]_{er}$ at the steady state was not observed when FCS was previously delipidated by charcoal treatment (data not shown).

Overexpression of $S1P_2$ or $S1P_3$ decreases pro-apoptotic effect of C_2-ceramide

In view of the protective role against C_2-ceramide-induced apoptosis exerted by reduced $[Ca^{2+}]_{er}$ levels [13], next the survival of native and S1PR-overexpressing HeLa cells treated with C_2-ceramide or staurosporine was investigated. For this purpose, HeLa cells were co-transfected with pcDNA3-$S1P_1$, or pcDNA3-$S1P_2$, or pcDNA3-$S1P_3$ and pEGFP. As it is illustrated in Fig. 4, by counting fluorescent transfected cells we found that in control and $S1P_1$-overexpressing cells the percentage of viable cells over the total cell population was the same before and after short chain ceramide addition, indicating that $S1P_1$ expression levels did not influence cell susceptibility to cell death. Conversely, cells overexpressing $S1P_2$, or $S1P_3$ were less sensitive to apoptosis, being the percentage of fluorescent cells over the total cell population higher than controls after C_2-ceramide addition ($25\% \pm 5$ vs $41\% \pm 8$ and $24\% \pm 4$ vs $37\% \pm 7$, respectively, $n = 3$; $p < 0.05$ in both cases) (Fig. 4A). After the treatment with staurosporine the percentage of fluorescent viable cells in $S1P_2$- and $S1P_3$-overexpressing cells was not different from that of native or $S1P_1$-overexpressing cells (Fig. 4B), indicating that the reduction of the free Ca^{2+} in the ER does not influence staurosporine-induced programmed cell death. Following both the treatments the number of overall surviving cells was reduced by approximately 90% by the apoptotic challenge (data not shown).

Discussion

In the past few years, it has been extensively demonstrated that S1P is a powerful sphingolipid capable of regulating many key biological events including cell proliferation, motility, and survival by the interaction with a set of specific receptors, which are almost ubiquitously expressed in higher organisms and result to be coupled to multiple classes of G proteins [2]. However, at present the molecular events triggered by the ligation of S1P to its receptors have not been fully elucidated. In this regard, although it is known that S1P induces Ca^{2+} transients in the cytosol in many cell types [6], no information is available about the kinetics of Ca^{2+} signaling in other intracellular organelles. Moreover, the implication of S1P-induced Ca^{2+} responses in its biological action has not been clearly addressed. The endogenous S1PRs expressed in HeLa cells were found not sufficient to induce detectable Ca^{2+} transients following S1P stimula-

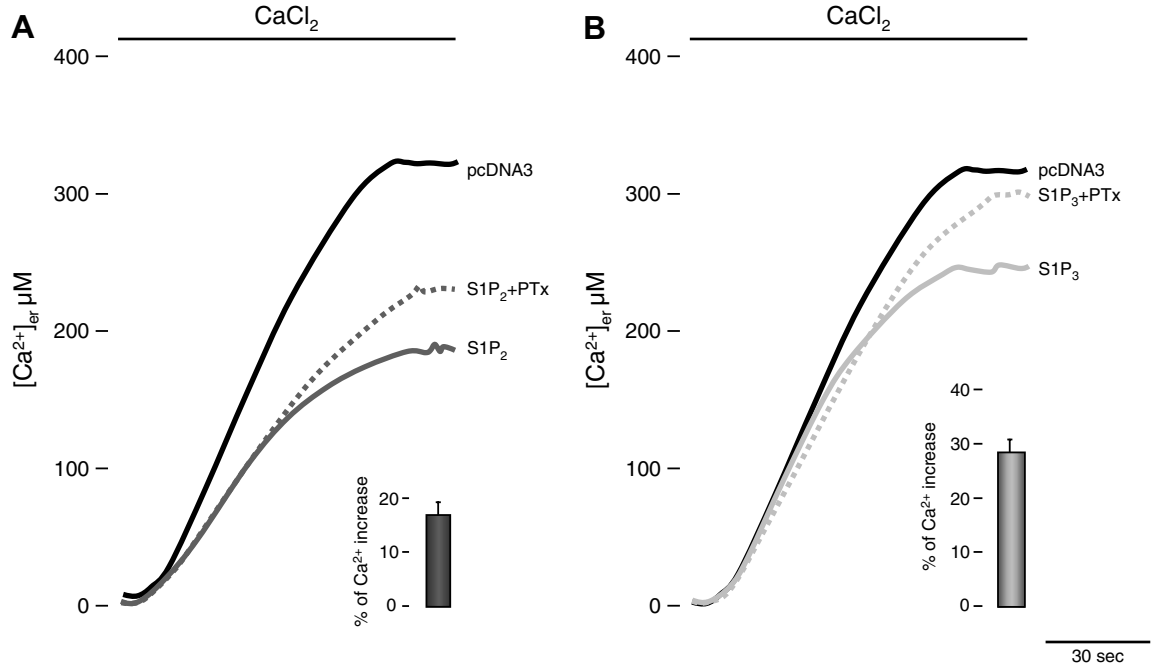

Fig. 3. Effect of PTx on ER Ca^{2+} homeostasis in HeLa cells overexpressing $S1P_2$, or $S1P_3$. $[Ca^{2+}]_{er}$ was measured in HeLa cells co-transfected with pcDNA3-$S1P_2$ (A) or pcDNA3-$S1P_3$ (B), and VR1012-erAEQ, treated for 16 h with 100 ng/ml PTx (gray and light gray dotted traces, respectively). After Ca^{2+} depletion in the ER, stores were refilled with Ca^{2+} by perfusing the cells with KRB containing 1.2 mM $CaCl_2$, until a plateau was reached. Traces are representative of >9 trials. Columns correspond to the percentage of the increase of $[Ca^{2+}]_{er}$ following PTx treatment.

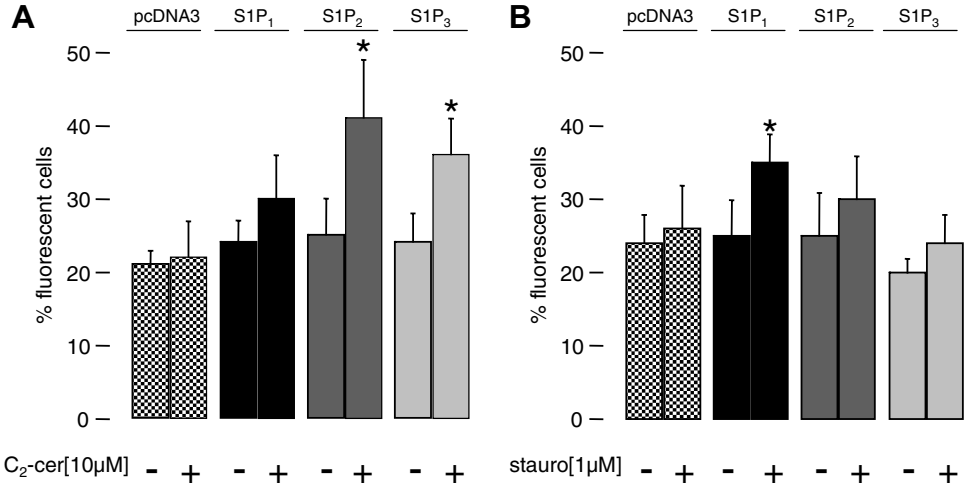

Fig. 4. Sensitivity to C_2-ceramide- and staurosporine-induced apoptosis of S1PR overexpressing HeLa cells. Hela cells co-transfected with pcDNA3-$S1P_1$, or pcDNA3-$S1P_2$, or pcDNA3-$S1P_3$, or pcDNA3 alone together with VR1012-erGFP were treated or not with 10 µM C_2-ceramide for 16 h (A) or 1 µM staurosporine for 4 h (B). Apoptotic response was evaluated by counting fluorescent surviving cells. Mean values were obtained from the analysis of >50 fields in three independent experiments ($p < 0.05$).

tion. Therefore, Ca^{2+} homoeostasis was investigated employing HeLa cells transiently overexpressing the individual S1PR subtypes together with aequorin. $S1P_1$ receptor subtype resulted to be uncoupled from $[Ca^{2+}]_{cyt}$ regulation, in agreement with several reports demonstrating that $S1P_1$ is not coupled to $[Ca^{2+}]_{cyt}$ increases [5,6]. Conversely, HeLa cells overexpressing $S1P_2$ or $S1P_3$, upon stimulation with S1P exhibited an increase in $[Ca^{2+}]$ in both the cytosol and mitochondria. Notably, when S1PR-overexpressing HeLa cells were examined for their responsiveness to histamine, which is a well-known regulator of Ca^{2+} homeostasis in these cells [14], native and $S1P_1$-overexpressing cells showed $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_m$ increases comparable to those previously reported [11,12] however, cytosolic and mitochondrial Ca^{2+} peaks elicited by the agonist were appreciably reduced in $S1P_2$- or $S1P_3$-overexpressing cells. The possibility that this feature was due to impairment in morphology and/

or distribution of mitochondria or ER in HeLa cells overexpressing $S1P_2$ or $S1P_3$ was excluded by microscopic analysis of intracellular organelles. Intriguingly, the investigation of $[Ca^{2+}]_{er}$ showed a significant difference in $S1P_2$-, or $S1P_3$-overexpressing cells in comparison with $S1P_1$-overexpressing or native cells, suggesting that the enforced expression of $S1P_2$ or $S1P_3$ impaired ER Ca^{2+} homeostasis. From data available in literature appears that intracellular Ca^{2+} increases induced by $S1P_2$ or $S1P_3$ engagement are at various extent sensitive to PTx, demonstrating that PLC activation and subsequent Ca^{2+} mobilization are mediated by G_q and to a lesser extent by G_i [6]. In this regard, when $S1P_2$- or $S1P_3$-overexpressing HeLa cells were treated with PTx a partial recovery in free $[Ca^{2+}]_{er}$ was observed, consistently with the hypothesis that the appreciable depletion of Ca^{2+} from the stores detected in $S1P_2$- or $S1P_3$-overexpressing cells is a receptor-mediated event resulting from the chronic stimulation of $S1P_2$ or $S1P_3$, brought about by S1P present in serum.

It has been demonstrated that $[Ca^{2+}]$ depletion in the ER is a key determinant of the susceptibility to programmed cell death induced by C_2-ceramide in HeLa cells [13]. In agreement, a major finding of this study is that HeLa cells expressing the recombinant $S1P_2$, or $S1P_3$ proteins were significantly less susceptible to C_2-ceramide-induced apoptosis than native or $S1P_1$-overexpressing cells. However, $S1P_2$- or $S1P_3$-overexpression was found unable to protect cells from staurosporine-induced apoptosis indicating that their sensitivity was dependent on the mechanism of action of the programmed cell death inducer. Since Ca^{2+} is a major effector of C_2-ceramide-induced apoptosis but is not implicated in the apoptotic response to staurosporine [15], these findings strongly support the hypothesis that the reduced $[Ca^{2+}]_{er}$ in $S1P_2$- and $S1P_3$-overexpressing cells is responsible for the observed protection from apoptosis in these cells.

One of the major current challenges in the field of sphingolipid cell biology is the understanding of the role played by the individual S1PR subtypes in cell signaling and the subsequent regulation of cell physiology. Indeed, each S1PR appears to be capable of activating distinct signaling pathways and usually multiple S1PR are simultaneously expressed in the same cell. In this vein, the present study represents an original contribution to the comprehension of the role exerted by individual S1PRs in the regulation of Ca^{2+} homeostasis. Moreover, these results provide also evidence for the implication of $S1P_2$- and $S1P_3$-dependent depletion of $[Ca^{2+}]_{er}$ in the protection from C_2-ceramide apoptosis. Since $S1P_2$ and $S1P_3$ expression levels appear to be regulated during embryonic development, cell differentiation and in response to growth factor and cytokine challenge in many cell types [2,16–18] it must be kept in mind that these processes imply also variations in cell sensitivity to certain apoptotic stimuli that can occur either in physiological or pathological conditions.

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