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Biochemical Pharmacology 64 (2002) 1243–1250

Biochemical Pharmacology

Comparative analysis of human and rat $S1P_5$ (edg8): differential expression profiles and sensitivities to antagonists

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Received 21 May 2002; accepted 6 July 2002

Abstract

Five guanine nucleotide-binding protein-coupled receptors ($S1P_{1–5}$) for the lysophospholipid mediator sphingosine 1-phosphate (S1P) have thus far been described. Whereas tissue distribution and functional properties of the human $S1P_{1–4}$ genes are well characterized, only limited functional and expression data are available for $S1P_5$, todate. Northern blot analysis indicated that human $S1P_5$ ($hS1P_5$) is an alternatively spliced gene, with a 5.4-kb transcript that is predominantly expressed in peripheral tissues, and a 2.4-kb transcript expressed in brain, spleen, and peripheral blood leucocytes. In contrast, rat $S1P_5$ ($rS1P_5$) was exclusively detected in brain and skin. Expression of $hS1P_5$ and $rS1P_5$ in mammalian CHO-K1 or HEK293 cells conferred onto the cells the ability to mobilize intracellular calcium as determined by a functional Fluorometric Imaging Plate Reader assay, when challenged with S1P and dihydro S1P, respectively. Applying a lipid library with 200 bioactive lipids in a functional Fluorometric Imaging Plate Reader assay did not reveal additional agonists. However, both receptors exhibited differential sensitivity towards the S1P- and lysophosphatidic acid-receptor antagonist, suramin: $rS1P_5$-mediated intracellular calcium mobilization was partly inhibited by suramin (IC_{50}: 5800 μM), whereas $hS1P_5$ was completely antagonized (IC_{50}: 130 μM). Both receptors were sensitive towards inhibition with the related drug (8,8′-(carbonylbis(imino-3,1-phenylene))bis(1,3,5-naphthalenetrisulfonic acid)) but IC_{50} values differed significantly (340 μM for $hS1P_5$, 4000 μM for $rS1P_5$). In addition, $rS1P_5$ displayed antiproliferative effects in transfected CHO-K1 and HEK293 cells in contrast to $hS1P_5$. Taken together, our data imply that differences between $hS1P_5$ and $rS1P_5$ will be an important point to be considered in the development of selective receptor antagonists.

Keywords: $S1P_5$ receptor; Expression profile; Sphingosine 1-phosphate; Lipid library; Suramin; NF023

1. Introduction

The lysolipid phosphate mediators, lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P), have attracted increasing attention as modulators of a variety of important biological functions [1–4] and their list of biological activities is continuously growing.

S1P has been implicated in cell proliferation, modulation of cell motility [5,6], induction/suppression of apoptosis [7,8], *in vitro* and *in vivo* angiogenesis [9], tumor invasiveness [10,11], platelet activation [12], and neurite retraction [13]. Cellular signaling by S1P involves activation of PLCβ and subsequent intracellular Ca^{2+} release [14,15], activation of MAP-kinases [16], activation of inward rectifying K^+-channels [17,18], and inhibition and/or activation of adenylyl cyclase [14].

Both LPA and S1P are recognized to signal cells through a set of G protein-coupled receptors (GPCRs), formerly known as endothelial differentiation gene (edg) receptors. This family of GPCRs currently comprises eight members and, on the basis of their activating ligand, can be classified into two major groups: $S1P_{1–5}$ [$S1P_1$ (edg1), $S1P_2$ (edg5), $S1P_3$ (edg3), $S1P_4$ (edg6), $S1P_5$ (edg8)] are stimulated by S1P [19–23], $LPA_{1–3}$ [LPA_1 (edg2), LPA_2 (edg4), LPA_3 (edg7)] preferentially interact with LPA [24,25].

Assignment of specific biological functions to certain receptor subtypes is hampered by: (1) the overlapping expression of S1P/LPA receptors [26,27]; (2) activation

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Abbreviations: S1P, sphingosine 1-phosphate; LPA, lysophosphatidic acid; dhS1P, dihydro sphingosine 1-phosphate; SPC, sphingosylphosphorylcholine; GPCR, G protein-coupled receptor; NF023, 8,8′-(carbonylbis(imino-3,1-phenylene))bis(1,3,5-naphthalenetrisulfonic acid); G protein, guanine nucleotide-binding protein; $[Ca^{2+}]_i$, intracellular calcium concentration; FCS, fetal calf serum; cs FCS, charcoal-stripped fetal calf serum; FLIPR, Fluorometric Imaging Plate Reader; PCR, polymerase chain reaction.

0006-2952/02/$ – see front matter
PII: S0006-2952(02)01289-3

of multiple, and in part redundant, signal transduction pathways [14–16,26]; (3) incomplete selectivity of their activating ligands [28,29]; and (4) poorly developed medicinal chemistry as specific antagonists for dissecting the pharmacology of individual subtypes are not yet available.

An important step to shed more light on the biological roles of the individual receptor subtypes would be to identify the complete set of receptors that respond to S1P and LPA and then to characterize the specific signaling and pharmacological properties.

Rat $S1P_5$ ($rS1P_5$) has recently been identified as the fifth S1P-responsive GPCR. It was originally cloned from rat pheochromocytoma cells, as a nerve growth factor regulated GPCR (nrg-1) [30] exhibiting greatest similarity to the family of $S1P_{1–4}$ receptors. $rS1P_5$ is expressed in spleen and adult brain white matter [23]. Malek *et al.* [28] demonstrated that $rS1P_5$ is coupled to G proteins of the $G_{i/o}$- and G_{12}-class, and inhibits activation of extracellular regulated kinase (ERK) in CHO cells. Much less is known about the human orthologue of $rS1P_5$. Im *et al.* [31] recently published preliminary expression and functional studies of human $S1P_5$ ($hS1P_5$). $hS1P_5$ is expressed in brain and peripheral tissues as determined by Northern blot analysis (multiple tissue dot blot); however, dot blot analysis revealed neither the transcript size nor the existence of tissue specific splice variants. In addition, low-level signals were detected in many tissues, making it hard to distinguish specific expression from background. Given the differential expression patterns of $hS1P_5$ and rodent $S1P_5$ receptors, we reasoned that additional pharmacological/biochemical differences may exist between the species homologues.

The present study was set out to address the following questions: (i) is $hS1P_5$ an ubiquitously or specifically expressed gene, (ii) how many splice variants exist for $hS1P_5$, and how are their tissue expression pattern, (iii) are the different expression patterns of $hS1P_5$ and $rS1P_5$ reflected in different sensitivities to agonists or antagonists, and (iv) does $hS1P_5$ resemble its rat counterpart in the antiproliferative activity upon expression in mammalian cell lines?

2. Materials and methods

2.1. Sources of materials

S1P, dhS1P, suramin, and fatty acid-free BSA were from Sigma. The lipid library was from Biomol Research Laboratories, Inc. NF023 was ordered from Tocris Cookson, CHO-K1 cells were obtained from the American Type Culture Collection, cell culture media and sera from Gibco BRL, the calcium fluorescent dye Fluo4 and pluronic acid from Molecular Devices, rat Northern blot membrane from Origene, human Northern blot membrane and the GC-melt PCR kit from Clontech. The expression vector PSPT18, the DIG-RNA Labeling kit and the DIG Wash and Block Buffer set were from Roche Diagnostics. Oligonucleotides from MWG-Biotech AG, the RT-PCR kit from Sigma, the expression plasmid pcDNA3.1 for $hS1P_5$ and $rS1P_5$ and pcDNA1.1 for expression of G protein α subunits, competent *Escherichia coli* DH5α from Gibco and *E. coli* MC 1061 from Invitrogen. Restriction enzymes and T_4-Ligase were from New England Biolabs.

2.2. Molecular cloning of the $hS1P_5$ and its rat homologue nrg-1 ($S1P_5$) receptor

As the $hS1P_5$ (AF317676) sequence is intronless, we cloned the receptor from human genomic DNA (Clontech) *via* polymerase chain reaction (PCR). PCR conditions were: denaturation (94° for 10 min), annealing (35 cycles of 94° for 1 min, 60° for 1 min), extension (72° for 2 min) using the GC-melt kit (Clontech). Primer contained a *Hin*dIII site in the forward, and an *Eco*RI-site in the reverse primer. The 1197 bp PCR product was cloned into a pcDNA3.1(+) mammalian expression vector and sequenced in both directions.

Rat nrg-1, acc. no. AF233649, was cloned from rat brain *via* RT-PCR. RNA was reversely transcribed into cDNA with the Prostar First Strand PCR Kit (Stratagene) as per manufacturer's protocol. The PCR reaction with the Advantage GC cDNA PCR Kit (Clontech) was performed as described for the $hS1P_5$ using primers amplifying the complete coding region from nucleotide 1 to 1203 with oligos carrying the restriction sites for *Hin*dIII in sense and for *Eco*RI in antisense direction. This fragment was cloned into the mammalian expression vector pcDNA3.1(+)Zeo and the plasmid was sequenced in both directions.

Murine wild type $G\alpha_q$ was cloned from mouse brain by RT-PCR and inserted into the *Bam*HI–*Nsi*I sites of pcDNA1.1. To create the C-terminally modified $G\alpha_{qi5}$ subunit, in which the last five aa of wt $G\alpha_q$ were replaced with the corresponding $G\alpha_i$ sequence, a 175-bp *Bgl*II–*Nsi*I fragment was replaced, in a two piece ligation, with a synthetic DNA fragment, containing the desired codon changes. The correctness of all PCR-derived sequences was verified by sequencing in both directions.

2.3. Northern blot analysis

Antisense RNA probes for $hS1P_5$ and $rS1P_5$ were generated by subcloning nucleotides 279–1197 ($hS1P_5$) or nucleotides 1–1203 ($rS1P_5$) of the coding region into the *Bam*HI–*Eco*RI sites of the expression vector PSPT18 and subsequent random priming with DIG-RNA Labeling kit using T_7 RNA polymerase. Hybridization was carried out at 68° for 16 hr in hybridization buffer (Dig Easy Hyb, Roche Diagnostics). The blot was washed, blocked, and detected as indicated in the standard protocol with the DIG Wash and Block Buffer set and treated with 1 mL ready-to-use CSPD (Roche Diagnostics) for 15 min (37°) and

developed for 5 min on the Lumiimager (Roche). Each blot was then stripped (50% formamid, 5% SDS, 50 mM Tris–HCl, pH 7.5, 80°, two times for 1 hr) and rehybridized with a DIG-labeled β-actin antisense RNA probe as an internal standard.

2.4. Cell culture and transfection

CHO-K1 cells were cultured at 37° in a humidified 5% CO_2 incubator in basal Iscove medium (Biochrom) supplemented with 10% fetal calf serum (FCS, Biochrom), penicillin–streptomycin (10,000 IU/mL to 10,000 μg/mL), Gentamicin (Roche), 2 mM L-Glutamin (Roche). Twenty-four hours after seeding of CHO-K1 cells (2×10^5/35 mm plate) at 50–80% confluency, the cells were transiently transfected with the indicated receptor and G protein constructs (1 μg of plasmid DNA each) using the LipofectAMINE Reagent (Gibco) as per manufacturer's instructions.

2.5. Fluorometric Imaging Plate Reader (FLIPR) assay

Eighteen to twenty-four hours after transfection, CHO-K1 cells were seeded into 96-well plates at a density of 60,000 cells per well and cultured for 18–24 additional hours until used in the functional FLIPR assays.

Cells were stained with Hank's balanced salt solution containing 20 mM HEPES, 2.5 mM probenecid, 4 μM fluorescent calcium indicator dye Fluo4, and 1% FCS for 1 hr (37°, 5% CO_2). Cells were washed three times with assay buffer (phosphate buffered saline (PBS), 1 mM $MgCl_2$, 1 mM EDTA, 0.4 mg/mL fatty acid-free BSA, 2.5 mM probenecid) in a Tecan cell washer. S1P and dhS1P were dissolved in DMSO as 2 mM stock solutions (treated with ultrasound when necessary), library compounds were provided by the supplier as 0.1 or 1.0 mM stock solutions in DMSO, except C_{16} ceramide that was dissolved in dimethylformamid. Library stock solutions were diluted 1:333 in 96-well plates and stored at −80° before use. All ligands were dissolved in assay buffer prior to addition to the cells. The FLIPR (Molecular Devices) was programmed to record fluorescence for a duration of 3 min as 1-s intervals during the first minute and 3-s intervals during the last 2 min. Peak fluorescence counts during the 18- to 37-s time points were used to determine agonist activity. Normalization of data was performed by instrument software.

For measurement of antagonist activity, suramin or NF023 were incubated with the cells for 3 min prior to addition of the agonist and fluorescence recorded as described above.

2.6. Proliferation assay

CHO-K1 cells (8×10^4) were seeded onto 35-mm dishes. After 32 hr, cells were transfected with 1 μg of the indicated plasmid ($hS1P_5$ in pcDNA3.1, $rS1P_5$ in pcDNA3.1, or pcDNA3.1 alone) using the LipofectAMINE Reagent according to the manufacturer's instructions. After 13 hr ($t = 0$), cells were once washed with PBS and grown for 48 hr in Iscove supplemented with 10% charcoal-stripped FCS (cs FCS, PAA Laboratories) in the presence or absence of 1 μM S1P.

Cells were counted at $t = 0$ and 48 hr using the Casy cellcounterTT (Schärfe System). Cells were first trypsinized with 400 μL trypsin and then resuspended in 1 mL medium. One hundred microliters of this cell suspension were diluted in 10 mL Casyton® and viable cells were measured with a 150 μm capillary in the cell counter. Each determination represents the mean ± SEM of three to four different wells.

3. Results

3.1. Expression of $hS1P_5$ and $rS1P_5$

Im *et al.* [31] recently showed, *via* dot blot analysis, that $hS1P_5$ is widespread in peripheral tissues and brain regions. To get more detailed information about the transcript size and putative splice variants, we examined the expression pattern of $hS1P_5$ by Northern blot analysis (Fig. 1A). A prominent band migrating at 5.5 kilobases (kb) was seen in skeletal muscle, heart, and kidney, while lower abundance of RNA was observed in liver and placenta; no signal was detected in brain, thymus, spleen, lung, and peripheral blood leukocytes. A second, less abundant transcript migrating at 2.4 kb was detected in brain, spleen, and peripheral blood leucocytes. Obviously, $hS1P_5$ exists in two splice variants, but only one splice variant can be detected at a time in a given tissue. We were able to confirm the results of Im *et al.* [23] that $rS1P_5$ is predominantly expressed in brain (Fig. 1B).

3.2. Functional properties of the $S1P_5$ receptor

$hS1P_5$ and $rS1P_5$ belong to the S1P receptor family [23] and are known to be activated by S1P and dhS1P. These two lipids unselectively activate the whole S1P receptor family [2,4], and thus are unsuitable for use in dissecting the pharmacology of S1P receptors. We sought to identify additional and perhaps more specific lipid agonists. To this end, we tested a library containing 201 bioactive lipids for agonist activity by measuring release of intracellular calcium ($[Ca^{2+}]_i$) with the FLIPR™ technology. The library covers several classes of bioactive lipids like prostaglandins, thromboxanes, leukotrienes, hydroxyeicosatetraenoic acids, dihydroxyeicosatetraenoic acids, lipoxins and other eicosanoids, unsaturated fatty acids, anandamides, retinoids, Vitamin D metabolites, proteasome proliferator activated receptor ligands, sphingoids, platelet activating factors and LPA (for a detailed list of lipids see supplementary data).

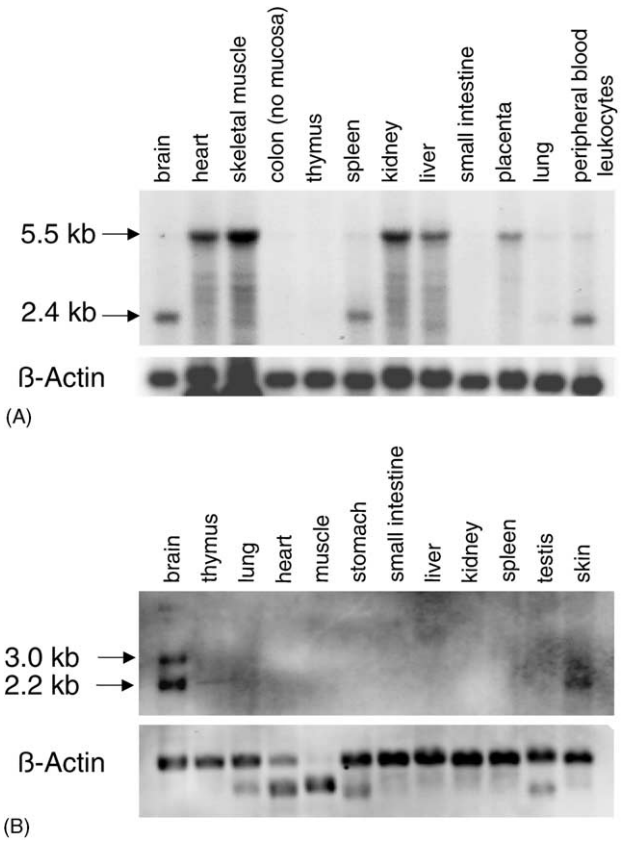

Fig. 1. Northern blot analysis of $S1P_5$ tissue distribution in several human (A) and rat (B) tissues. Poly(A)+ RNA from various human (1 μg) and rat tissues (2 μg) was hybridized with DIG-labeled probes specific to human (A) or rat (B) $S1P_5$ on a nylon membrane. Each blot was stripped and reprobed for β-actin. The origin of each RNA is indicated at the top, the molecular mass of the detected bands in kilobases (kb) is shown on the left.

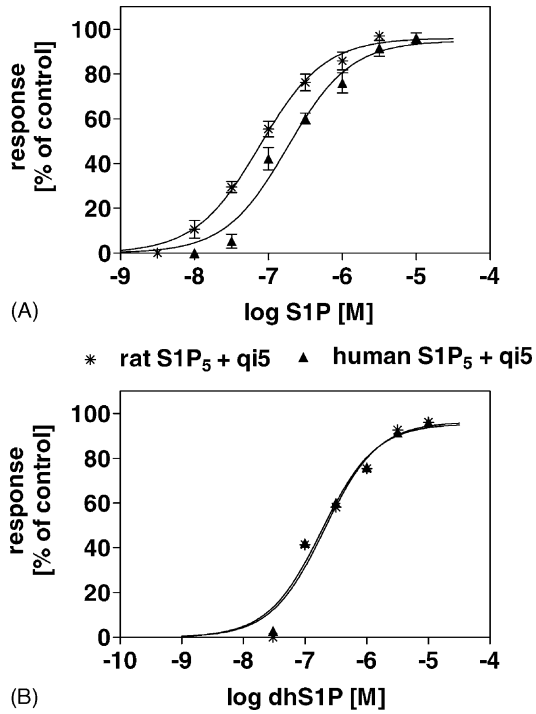

Fig. 2. S1P- (A) and dhS1P- (B) mediated $[Ca^{2+}]_i$ release in CHO-K1 cells transiently cotransfected with $hS1P_5$ or $rS1P_5$ and $G\alpha_{qi5}$. CHO-K1 cells transiently transfected with the indicated receptor and $G\alpha_{qi5}$ were plated into 96-well plates, grown for 24 hr, loaded with the fluorescent calcium indicator Fluo4 and then stimulated with increasing concentrations of S1P (A) or dhS1P (B). $[Ca^{2+}]_i$ increases were measured with the FLIPR™ technology as described in detail in the experimental section. Peak fluorescence counts were normalized and maximum responses were set to 100%. Data are means ± SEM of four to six independent experiments.

We transiently cotransfected mammalian CHO-K1 cells—as they are in contrast to HEK293 or COS cells known to exhibit negligible S1P-binding, when vector-transfected [28]—with the cDNAs for $hS1P_5$ and $rS1P_5$, and the chimeric G protein $G\alpha_{qi5}$, which confers onto G_i-coupled receptors the ability to stimulate the G_q-pathway [32], and thus to promote coupling to intracellular calcium. It should be noted that in CHO-K1 cells the evocation of calcium responses required cotransfection of $S1P_5$ and chimeric $G\alpha_{qi5}$. CHO-K1 cells transfected with chimeric $G\alpha_{qi5}$ alone were unresponsive to any lipid applied at 1 μM concentration, apart from LPA. Only cells transfected with both $S1P_5$ and $G\alpha_{qi5}$ were responsive to S1P and dhS1P. None of the other lipids tested displayed any agonist activity on $hS1P_5$ or $rS1P_5$ that was detectable beyond background.

Fig. 2A and B display dose–response curves of S1P- and dhS1P-mediated increases of $[Ca^{2+}]_i$ in CHO-K1 cells transiently transfected with $S1P_5$ receptor and $G\alpha_{qi5}$ cDNAs. The EC_{50} for S1P on $hS1P_5$ and $rS1P_5$ was 1.8×10^{-7} and 7.8×10^{-8} M (Fig. 2A), respectively; dhS1P displayed EC_{50} values of 1.8×10^{-7} and 2.1×10^{-7} M on $hS1P_5$ and $rS1P_5$, respectively (Fig. 2B).

We also investigated the effects of the polycyclic anionic compound suramin and its analogue, NF023, on S1P-mediated receptor activation. Suramin is known to block many receptor–ligand interactions including those of S1P and LPA [13,33], and has recently been reported to be a selective $S1P_3$ receptor antagonist that does not inhibit $S1P_1$ and $S1P_2$ [15] in functional assays using *Xenopus* oocytes. In this study [15], $hS1P_5$ and $rS1P_5$ were not included and the effects of suramin and NF023 on $S1P_5$ receptors have not been examined to date.

To determine the antagonistic properties of suramin and NF023 on S1P-mediated $[Ca^{2+}]_i$ mobilization, CHO-K1 cells expressing $hS1P_5$ or $rS1P_5$ and $G\alpha_{qi5}$ were incubated with increasing concentrations of suramin or NF023. The cells were then challenged with S1P, and calcium responses were recorded. Suramin concentrations were chosen according to a recent publication by Ancellin and Hla [15], where suramin was applied in concentrations up to 3000 μM. Interestingly, $hS1P_5$ was sensitive to suramin and NF023 antagonism: S1P-mediated calcium release was almost completely blocked in the presence of 3000 μM suramin (Fig. 3A) as well as in the presence of 10,000 μM NF023 (Fig. 3B). IC_{50} values for inhibition of

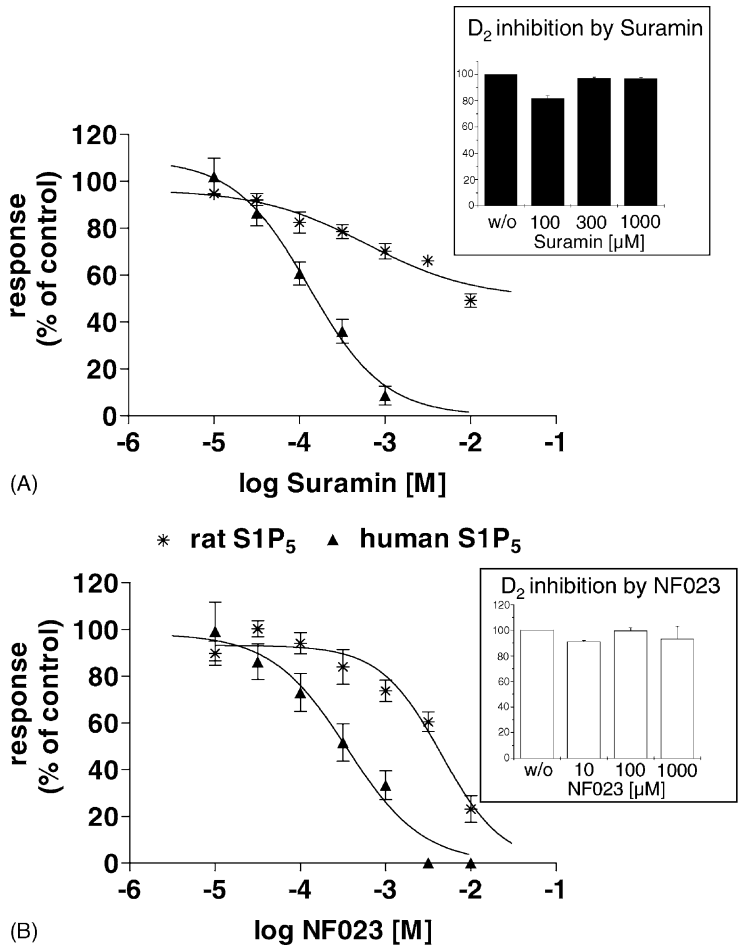

Fig. 3. Inhibition of S1P-mediated intracellular calcium release by suramin (A) and its analogue NF023 (B) in CHO cells transiently cotransfected with $hS1P_5$ or $rS1P_5$ and $G\alpha_{qi5}$. The FLIPR assay was carried out as described in Fig. 2. Essentially, for measurement of antagonistic effects, transfected cells were first treated with the indicated concentrations of the inhibitor or solvent buffer for 3 min (NF023 and suramin did not show any effect on $[Ca^{2+}]_i$ mobilization during the preincubation period). Cells were then stimulated with 3 µM S1P ($hS1P_5$) or 1 µM S1P ($rS1P_5$) and $[Ca^{2+}]_i$ measured with the FLIPR. Concentrations of S1P for each receptor correspond to 85% of the maximum response. Peak fluorescence counts were normalized. S1P-mediated calcium release in the absence of inhibitor was set 100%. Data are means ± SEM of four to six independent experiments. Insets: inhibition of Dopamin-mediated $[Ca^{2+}]_i$ release by suramin (A) or NF023 (B) in CHO-K1 cells transiently cotransfected with human D_2 receptor and $G\alpha_{qi5}$. Cells were stimulated with 100 nM dopamine and calcium release recorded essentially as described above. Data are means ± SEM of three independent experiments.

S1P-induced calcium release were 1.3×10^{-4} and 3.4×10^{-4} M for suramin and NF023, respectively (Fig. 3A and B). $rS1P_5$-mediated $[Ca^{2+}]_i$ mobilization was only partially responsive to suramin, with an IC_{50} value of 5.8×10^{-3} M and a maximum inhibition of 50% (Fig. 3A). NF023 completely blocked $rS1P_5$ induced $[Ca^{2+}]_i$ release with an IC_{50} value of 4×10^{-3} M, which is about 10-fold higher than that for the $hS1P_5$ (Fig. 4B).

In order to find out whether the antagonistic effects of suramin and NF023 on $hS1P_5$ and $rS1P_5$ were due to nonspecific effects on receptor–G protein coupling, or to specific inhibition of the transmembrane receptor, the following control experiment was performed: the G_i-coupled dopamine D_2 receptor, when coexpressed with $G\alpha_{qi5}$, was neither antagonized by suramin (inset Fig. 3A) nor by NF023 (inset Fig. 3B), ruling out nonspecific effects of receptor–G protein coupling by suramin and NF023.

3.3. $hS1P_5$ effects on cell proliferation

S1P has been shown to stimulate proliferation through $S1P_2$, $S1P_3$, and possibly $S1P_1$ [34–36]. Malek *et al.* [28] recently reported on the S1P-induced inhibitory effects of $rS1P_5$ on cell proliferation in transiently transfected CHO-K1 cells. We, therefore, investigated whether the human homologue resembles its rat counterpart with respect to the antiproliferative effects in transfected CHO-K1 cells. $rS1P_5$ was included as a positive control in each assay to allow for direct comparison and assay conditions were essentially as described in [28]. As expected, $rS1P_5$ significantly inhibited cell proliferation, when stimulated with 1 µM S1P for 48 hr (Fig. 4). In contrast, $hS1P_5$ failed to exhibit antiproliferative effects. $rS1P_5$ and $hS1P_5$ did not display intrinsic antiproliferative effects. The antiproliferative responses of S1P acting *via* the two GPCRs were

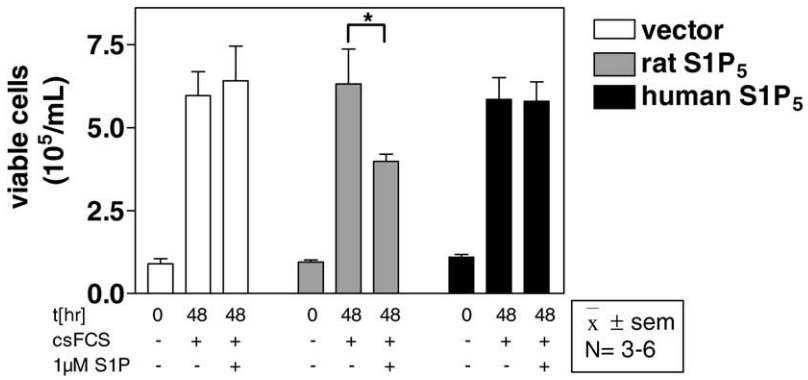

Fig. 4. Effects of $hS1P_5$ and $rS1P_5$ on proliferation of transiently transfected CHO-K1 cells. CHO-K1 cells (8×10^4) were seeded onto 35-mm dishes. After 32 hr, cells were transfected with the indicated plasmid ($hS1P_5$, $rS1P_5$, or vector alone). After 13 hr ($t = 0$), cells were washed once with PBS and grown for 48 hr in Iscove supplemented with 10% cs FCS in presence or absence of 1 μM S1P. Cell proliferation was quantitated as described under Section 2. Data represent the mean ± SEM of three to six different wells and are expressed as viable cells per milliliter. An asterisk (∗) indicates a statistically significant difference, as determined by the Student's *t*-test ($P < 0.05$).

initially determined after different time intervals (24, 36, and 48 hr). Twenty-four hours after stimulation with S1P, antiproliferative effects were barely detectable for $rS1P_5$ (data not shown). They were slightly increased 36 hr after stimulation (data not shown), but very significant 48 hr after S1P stimulation.

Very similar results were obtained from $S1P_5$ transfected HEK293 cells: neither receptor displayed agonist-independent antiproliferative effects, but $rS1P_5$ did exhibit antimitogenic effects (data not shown), when incubated with 1 μM S1P for 48 hr.

4. Discussion

Recently, $hS1P_5$ has been discovered as the fifth member of the S1P receptor family [31]. Whereas many S1P receptors are ubiquitously expressed [27,37,38], $hS1P_5$ displays a more specific expression pattern. Northern blot analysis of this study has revealed two new and interesting aspects of its expression: (1) $hS1P_5$ is mainly expressed in the cardiovascular system, as opposed to $rS1P_5$, that is exclusively expressed in brain, spleen, and skin; (2) $hS1P_5$ exists as two splice variants with one transcript occurring only in peripheral tissues, and a second less prominent transcript in brain, spleen, and peripheral blood leukocytes. These differences in the expression profiles of $hS1P_5$ and $rS1P_5$ despite their high homology suggests that the receptors may play different roles in human and rat physiology. The $rS1P_5$ is probably a member of the S1P/LPA-family, exerting its major effects in the CNS, and the human orthologue may be implicated in regulating cardiovascular homeostasis. These discrepancies are so far unique for the two members of the S1P/LPA-family of GPCRs, and imply that care has to be taken when interpreting data on $S1P_5$-mediated responses in animal models. In addition, Im *et al.* [31] reported the mouse $S1P_5$ expression profile to be very similar to $rS1P_5$ suggesting a general difference between humans and rodents.

Given the significant species differences in $S1P_5$ receptor expression, we were tempted to speculate about pharmacological differences regarding, for example, responsiveness to different agonists. To date, S1P and dhS1P have been identified as ligands for $S1P_5$ receptors. The literature on GPCRs supports the possibility of activation by multiple ligands. Zhu *et al.* [39] have shown that the orphan receptor GPR4 responds to sphingosylphosphorylcholine (SPC) and lysophosphatidylcholine (LPC), whereas GPR68—also known as ovarian cancer GPCR "OGR1"—exclusively responds to SPC [40]. The corticotrophin releasing factor (CRF) receptor family is another example of GPCRs activated by a plethora of different ligands [41,42]. Thus, to test for potential ligand diversity within the S1P receptor family, we applied a library of bioactive lipids containing 201 potential agonists; structurally the compounds ranged from the almost-identical to the completely different, as compared to S1P. We did not identify any difference regarding the profiles of activating ligands. S1P and dhS1P were active on both receptors, suggesting a high specifity of the receptor for the structural features of these lysophospholipids. The fact that we could not identify SPC as an activating ligand, as described by Im *et al.* [23] using GTPγS binding assays, may be due to the different cell types used: HEK293, RH7777 as opposed to CHO-K1 in our study. Malek e*t al.* [28] corroborated these data as they failed to detect SPC binding in CHO-K1 cells. Alternatively, as it is known that activation of $S1P_5$ receptors requires rather high concentrations of SPC (EC_{50} ~1 μM in GTPγS assays with very low efficacy [23]), it may well be that the chosen concentration of SPC (1 μM) did not suffice for a calcium response in our functional assay.

So far, only very close structural analogues of S1P—the chiral 3-hydroxy function was replaced with an amide or carbonyl group—synthesized by Im *et al.* [31] are able to

stimulate GTPγS binding on $S1P_5$ expressing membranes, *albeit* with a lower potency than S1P. It remains to be tested whether these lipids also evoke functional responses in different second messenger assays or *in vivo*. In addition, it should be noted that the lipids in our system were used at concentrations from 100 nM to 1 μM, a range in which most of the compounds tested by Im *et al.* [31] were still inactive or only generated a small signal. Thus, it may be possible that we failed to identify additional activating ligands due to insufficient potency. As our goal was the identification of specific and potent agonists, compounds were applied in a concentration range suitable for physiological ligand–receptor interactions.

We next wanted to determine the effects of suramin and its analogue, NF023, on S1P-induced calcium mobilization *via* $hS1P_5$ and $rS1P_5$. Suramin-induced inhibition of $hS1P_5$- or $rS1P_5$-mediated functional responses has not yet been described in the literature. Our results reveal that $hS1P_5$ is clearly more susceptible to inhibition by suramin or NF023 than is the rat orthologue (Fig. 3). Additionally, our results indicate that suramin can no longer be regarded as a selective $S1P_3$ receptor antagonist as it does also inhibit $hS1P_5$ and to a lesser extent $rS1P_5$. The actions of suramin and NF023, in our assay system, seem to be directed at blocking receptor–ligand interaction, rather than being a nonspecific antagonist of receptor–G protein coupling: (i) $hS1P_5$ is clearly more susceptible to inhibition by suramin or NF023 than $rS1P_5$ (Fig. 4), (ii) the extent of $rS1P_5$ inhibition in HEK293 cells was identical whether $G\alpha_{qi5}$ was coexpressed or not (data not shown), (iii) $hS1P_5$ was antagonized by suramin in HEK293 cells whether $G\alpha_{qi5}$ was coexpressed or not (data not shown), (iv) the G_i-coupled dopamine D_2 receptor, when coexpressed with $G\alpha_{qi5}$, was neither inhibited by suramin nor NF023 (insets Fig. 3).

Species specific antagonism of suramin and NF023, preferentially inhibiting $hS1P_5$ but not $rS1P_5$, is surprising as the homology between both receptors is quite high (∼87%). Nevertheless, variations in receptor structures exist and may be sufficient to account for the observed differences in sensitivity towards the antagonists. This crucial finding should be considered, when developing selective $S1P_5$ receptor antagonists, as they may act differentially on $S1P_5$ receptors in various species.

Last but not least, it is intriguing to note that rat but not $hS1P_5$ expression in mammalian cells exerts antiproliferative effects, supporting the notion that both receptors may play different physiological/pathophysiological roles. It would be interesting to determine the cause of the observed differences, as the upstream signal transduction events initiated by both receptors are identical (inhibition of adenylyl cyclase *via* $G\alpha_i$ type of G proteins). Downstream divergence of signaling, probably on the level of MAP kinases may account for the opposing proliferative responses. This is the first study to demonstrate that two S1P receptor species homologues may differ significantly in their tissue distribution and in their pharmacological/cell biological properties, which is so far unique to the S1P family of GPCRs.

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