Photolysis of intracellular caged sphingosine-1-phosphate causes Ca^{2+} mobilization independently of G-protein-coupled receptors

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Abstract Sphingosine-1-phosphate (S1P), the product of sphingosine kinase, activates several widely expressed G-protein-coupled receptors (GPCR). S1P might also play a role as second messenger, but this hypothesis has been challenged by recent findings. Here we demonstrate that intracellular S1P can mobilize Ca^{2+} in intact cells independently of S1P-GPCR. Within seconds, S1P generated by the photolysis of caged S1P raised the intracellular free Ca^{2+} concentration in HEK-293, SKNMC and HepG2 cells, in which the response to extracellularly applied S1P was either blocked or absent. Ca^{2+} transients induced by photolysis of caged S1P were caused by Ca^{2+} mobilization from thapsigargin-sensitive stores. These results provide direct evidence for a true intracellular action of S1P.

Key words: Ca^{2+} mobilization; Photolysis; Sphingosine-1-phosphate; Thapsigargin-sensitive Ca^{2+} store

1. Introduction

Sphingosine-1-phosphate (S1P) is a key signalling molecule regulating cell growth, migration, programmed cell death and development in organisms from yeast, plant, worms and flies to mammals (reviewed in [1–4]). It is well established that many of the diverse actions of S1P are mediated by S1P-specific G-protein-coupled receptors (S1P-GPCR) [5–8]. Five of the S1P-GPCR belong to the EDG family of receptors, they were recently renamed as $S1P_{1-5}$ [9], while three newly identified receptors are not related [10]. The S1P-GPCR are nearly ubiquitously expressed and regulate many cellular functions, for example cell proliferation, organization of the cytoskeleton, cell migration, increases in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), cardiovascular development and immune responses [1,6].

One of the most controversial questions in the field of S1P signalling is whether S1P can also act intracellularly [3,4,6,11]. Cellular levels of S1P are tightly regulated by sphingosine kinase, S1P lyase and phosphatases (for review see [4]). It has been suggested that the balance of ceramide and S1P inside the cell forms a rheostat decisive for cell growth or death [4]. Furthermore, intracellular S1P has been implicated in Ca^{2+} mobilization [12]. However, it was recently demonstrated that S1P as well as sphingosine kinase can be secreted [13,14]. Furthermore, cell migration stimulated by the platelet-derived growth factor receptor, which is dependent on the activation of sphingosine kinase, was blocked by knockout of the $S1P_1$ receptor, indicating that at least in this system the intracellularly generated S1P acts via a S1P-GPCR [13,15].

We and others have previously shown that diverse plasma membrane receptors rapidly stimulate intracellular S1P production, and that inhibition of sphingosine kinase attenuated $[Ca^{2+}]_i$ increases by these receptors [16–19]. To claim that S1P indeed acts as a Ca^{2+}-mobilizing second messenger in these cellular systems, it is necessary to prove that S1P can mobilize Ca^{2+} by acting intracellularly. Studies on Ca^{2+} mobilization by intracellular S1P are hampered by several problems. First, in contrast to the initial report of S1P-induced Ca^{2+} release in permeabilized smooth muscle cells and microsomes [20], subsequent studies found little if any effects of S1P on Ca^{2+} release in similar in vitro systems, whereas the related lipid sphingosylphosphorylcholine was active [18,21,22] (D.M.z.H., unpublished observations). Second, several of the ubiquitously expressed S1P-GPCR couple to intracellular Ca^{2+} mobilization when activated by extracellular S1P [7,8,10]. Third, although microinjection of S1P induced intracellular Ca^{2+} release [16], this procedure requires damage of the plasma membrane, and high S1P concentrations in the injection solution may also perturb intracellular membranes. To overcome these difficulties, we explored the release of S1P from caged S1P by photolysis. This technique provides an ideal approach to study Ca^{2+} release by intracellular S1P, because it maintains the intact cellular milieu that is apparently required.

The synthesis of caged S1P and its use to monitor possible intracellular S1P effects on DNA synthesis have been reported previously [23]. However, the cells used in that study responded to extracellular S1P, thus confounding extracellular actions of the photo-released S1P could not be excluded. Here

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Abbreviations: $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; GPCR, G-protein-coupled receptor; HBSS, Hank's balanced salt solution; I_{Cl}, chloride current; IP_3, inositol-1,4,5-trisphosphate; PTX, pertussis toxin; S1P, sphingosine-1-phosphate

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we used photolysis of caged S1P to demonstrate that intracellular S1P releases Ca^{2+} in various cell types in which an action of extracellular S1P on $[Ca^{2+}]_i$ was either pharmacologically blocked or absent. These results provide the evidence for a true intracellular action of S1P.

2. Materials and methods

2.1. Cell culture

HEK-293 cells stably expressing the M_3 muscarinic acetylcholine receptor [24] were cultured as described before [16]. HepG2 cells and SKNMC cells were grown in Dulbecco's modified Eagle's medium (DMEM/F12), supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and 0.1 mg/ml streptomycin, in a humidified atmosphere of 5% CO_2/95% air at 37°C. Prior to experiments, the cells were seeded onto glass coverslips that were either coated with 12.5 μg/ml poly-L-lysine (HEK-293 cells) or uncoated (HepG2 cells, SKNMC cells). Where indicated, HEK-293 cells were treated for 24–48 h with 100 ng/ml pertussis toxin (PTX).

2.2. Ca^{2+} imaging and photolysis

$[Ca^{2+}]_i$ was measured by imaging the fluorescence of the Ca^{2+} indicator dye, fluo-4. The cells were washed with Hank's balanced salt solution (HBSS; 118 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose and 15 mM HEPES, pH 7.4) and loaded with 4 μM fluo-4/AM (Molecular Probes; www.probes.com) in HBSS for 30 min at room temperature. After loading, the cells were washed with HBSS, and the experiments were performed at room temperature. Ca^{2+} imaging was done using an inverted confocal laser scanning microscope (Zeiss LSM510) and a 40×/1.3 Plan-Neofluar objective (Carl Zeiss MicroImaging, Inc.). The dye was excited with the 488 nm line of an argon laser, and emission was recorded with a 505 nm long pass filter. Caged S1P (Alexis Biochemicals; www.alexis-corp.com) was dissolved at 10 mM in dimethylsulfoxide and further diluted in HBSS containing 1 mg/ml fatty acid-free bovine serum albumin. The final dimethylsulfoxide (DMSO) concentration never exceeded 0.4% and DMSO up to 1% did not have any influence on Ca^{2+} signalling. For intracellular S1P generation, the cells were loaded with caged S1P for a minimum of 10–30 min. Photolysis was performed by brief light pulses of the 364 nm line of an argon laser (Enterprise II, 80 mW, Laser Innovations) with the maximal intensity. The timing and the duration of the laser pulses were recorded in a separate channel as marked on the figures. In most experiments, fluorescence images were collected from an area of ~60×60 μm at a frequency of two per second and a spatial resolution of 128×128 pixel. Mean fluorescence of single cells was calculated by averaging regions of interest selected by drawing around the individual cells. The individual traces shown in the figures represent time courses of $[Ca^{2+}]_i$ of a single cell each. Cells within one figure were from one microscopic field. All experiments were performed three to 20 times in different microscopic fields with similar results.

3. Results and discussion

First, we monitored photolysis-induced generation of S1P by measuring $[Ca^{2+}]_i$ elevations mediated by the highly sensitive S1P-GPCR present in HEK-293 cells, which express the $S1P_1$, $S1P_2$ and $S1P_3$ receptor subtypes [25]. In these cells, extracellularly applied S1P elicits a transient rise in intracellular Ca^{2+} with an EC_{50} of ~2 nM [26]. A brief (~6 s) exposure of the cells to the 364 nm line of an argon laser in

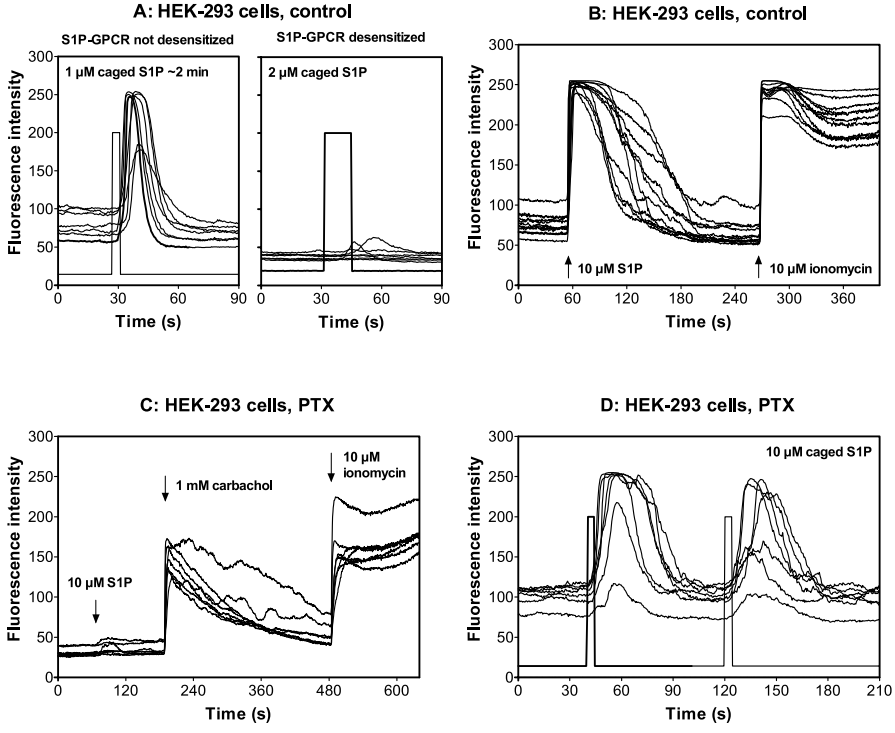

Fig. 1. Generation of bioactive S1P by photolysis of caged S1P, and stimulation of $[Ca^{2+}]_i$ increase by intracellular S1P liberation in PTX-treated HEK-293 cells. $[Ca^{2+}]_i$ was measured by fluorescence imaging of fluo-4-loaded cells as described in Section 2. The traces represent the mean fluorescence of individual cells. The rectangular lines in panels A and D mark the time points at which the 364 nm UV laser line used for uncaging was switched on and off. Traces within one panel were from one microscopic field. A: Response to photolysis of 1–2 μM caged S1P in control HEK-293 cells expressing the S1P-GPCR, $S1P_1$, $S1P_2$ and $S1P_3$. In the right panel, the S1P-GPCR had been desensitized by pretreatment with 2 μM extracellular S1P. B,C: Responses of control and PTX-treated HEK-293 cells to 10 μM extracellular S1P, 10 μM ionomycin and 1 mM carbachol, as indicated by the arrows. D: Response of PTX-treated HEK-293 cells to photolysis of caged S1P. The cells was loaded for 10 min with 10 μM caged S1P. E: Time course of fluorescence images corresponding to panel D. The numbers given in the left upper corners mark the time points (in seconds) at which the images were taken. The asterisks mark the application of the UV laser light. For clarity only every fourth image taken is shown here. The frame size in this experiment was 110×110 μm.

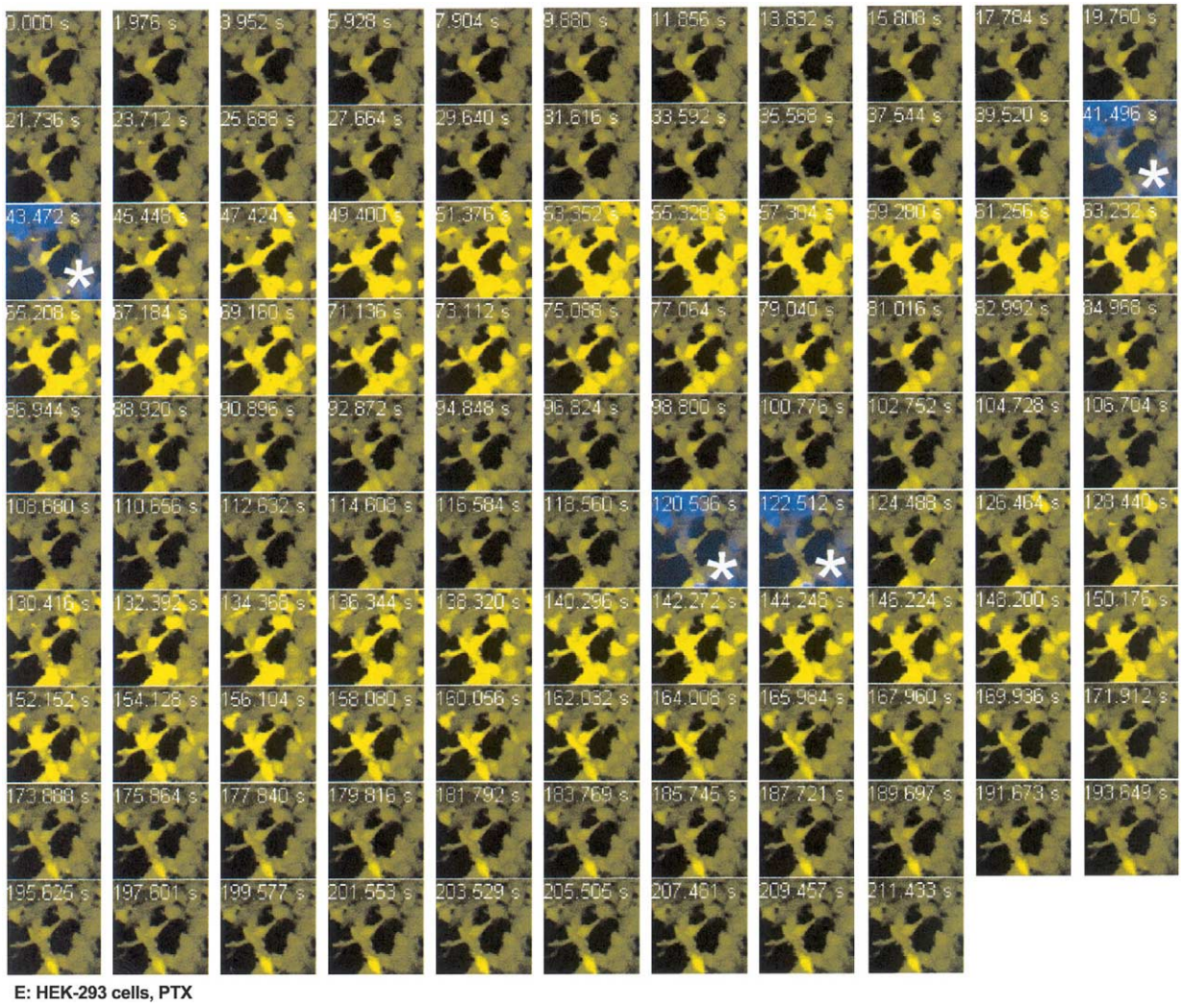

Fig. 1 (*Continued*).

the presence of 1–2 μM caged S1P elicited transient $[Ca^{2+}]_i$ increases, measured with the $[Ca^{2+}]_i$ indicator dye fluo-4 (Fig. 1A). This response was not observed in cells in which the S1P-GPCR were desensitized by prior extracellular application of S1P (Fig. 1A), as repeated application of S1P causes rapid, near-complete desensitization in these cells [26]. These data demonstrate that bioactive S1P was rapidly generated from the caged compound by brief pulses of the UV laser.

To address the question whether caged S1P entered the cells and induced intracellular Ca^{2+} release independently of S1P-GPCR upon photolysis, we treated the HEK-293 cells with PTX, because $[Ca^{2+}]_i$ transients elicited by the endogenous S1P-GPCR in HEK-293 cells are mediated by G_i-proteins [16,26]. As illustrated in Fig. 1B, application of 10 μM S1P, which is a supramaximal concentration for activation of S1P-GPCR, rapidly increased $[Ca^{2+}]_i$ in control HEK-293 cells. In contrast, pretreatment with PTX abolished the response even to this high S1P concentration in most of the cells, with only few cells showing a minor increase in $[Ca^{2+}]_i$ (Fig. 1C), while $[Ca^{2+}]_i$ increase mediated by the G_q-coupled M_3 muscarinic receptor expressed in these cells was not affected by PTX (Fig. 1C). UV laser pulses did not increase fluo-4 fluorescence or $[Ca^{2+}]_i$ in the absence of caged S1P (data not shown). When PTX-treated HEK-293 cells were incubated with 10 μM caged S1P, brief (~6–8 s) UV laser pulses within seconds induced transient increases in $[Ca^{2+}]_i$ (Fig. 1D,E). Because extracellularly applied S1P was ineffective under these conditions (Fig. 1C), such an elevation in $[Ca^{2+}]_i$ is consistent with an intracellular, non-GPCR-mediated action of S1P.

The following four observations further support an intracellular non-S1P-GPCR-mediated action of photoreleased S1P: First, photolysis of caged S1P elevated $[Ca^{2+}]_i$ even in the continuous presence of 1 μM extracellular S1P (Fig. 2A), which causes desensitization of S1P-GPCR (Fig. 1A and [26]). Second, photolysis increased $[Ca^{2+}]_i$ after removing caged S1P from the bath (Fig. 2B). Caged S1P appears to be rapidly metabolized or extruded from the cells because after ~5 min of washing the response began to decline (data not shown). Third, time-course experiments revealed that $[Ca^{2+}]_i$ transients induced by photolysis of caged S1P in PTX-treated cells occurred only after a minimum loading time of ~5–8 min (Fig. 2C), which was not required for the response in control cells (Fig. 1A). Finally, high concentrations of caged S1P (10–40 μM) were required to elicit $[Ca^{2+}]_i$ transients in PTX-treated

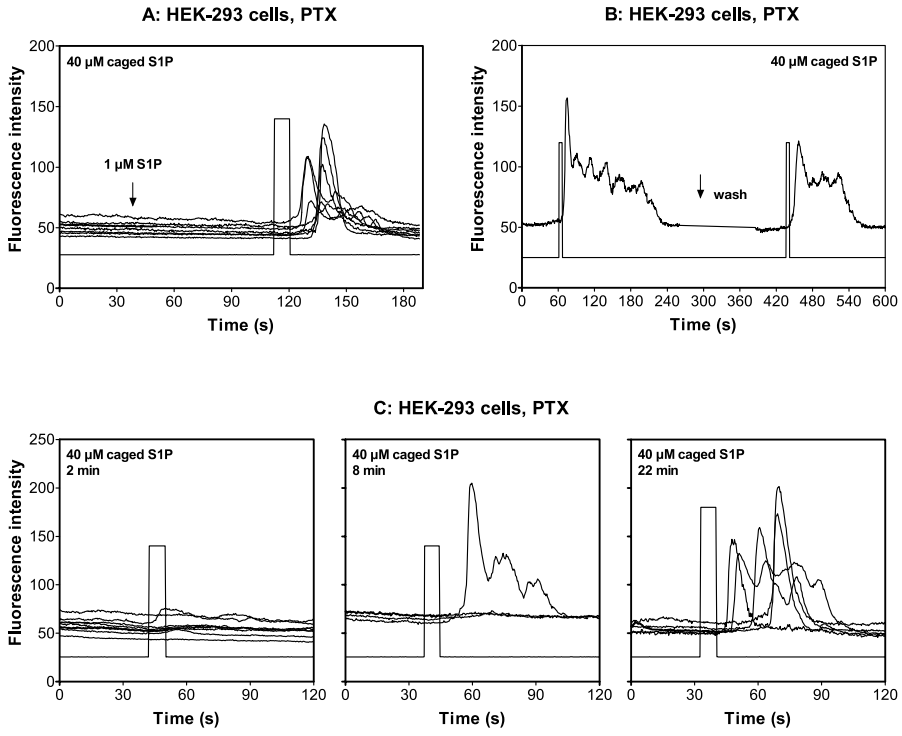

Fig. 2. Stimulation of $[Ca^{2+}]_i$ increase by intracellular S1P generation in PTX-treated HEK-293 cells. A: Response of seven cells to photolysis of caged S1P after addition of 1 µM extracellular S1P. The cells were loaded for 30 min with caged S1P. B: Effect of removal of extracellular caged S1P. Cells loaded with caged S1P for 45 min were exposed to UV laser pulses before and shortly after removal of caged S1P from the bath solution. For clarity, the response of one cell out of four with similar behavior is shown. C: Response of PTX-treated HEK-293 cells to photolysis of caged S1P after loading with caged S1P for different periods of time.

HEK-293 cells. This is in sharp contrast to the 1–2 µM concentration of caged S1P, decaging of which was sufficient to elicit activation of S1P-GPCR in control cells (Fig. 1A) but was ineffective in PTX-treated cells (data not shown). Taken together, these data indicate that the lipophilic caged S1P crosses the plasma membrane and that minutes are required to reach an effective intracellular concentration. $[Ca^{2+}]_i$ increases induced by photolysis of caged S1P were also dependent upon the intensity and duration of the UV laser pulse (data not shown). Thus, Ca^{2+} mobilization by intracellular S1P was a concentration-dependent effect. Because neither loading efficiency nor intracellular decaging efficiency could be accurately determined using the intact cells, we were unable to estimate the concentrations of intracellular S1P required for threshold and maximum Ca^{2+} mobilization.

To corroborate the finding that intracellular S1P mobilizes Ca^{2+} independently of S1P-GPCR, it was important to demonstrate this effect in cells which do not endogenously respond to extracellularly applied S1P. To date, eight distinct S1P-GPCR have been identified [5,10], and more might exist (see e.g. [27]). We identified two human cell lines that do not respond to extracellularly applied S1P with $[Ca^{2+}]_i$ transients, the human neuroepithelioma cell line SKNMC and the human hepatocellular carcinoma cell line HepG2. As shown in Figs. 3A and 4A, extracellularly applied S1P or brief UV laser pulses (not shown) did not induce detectable changes in $[Ca^{2+}]_i$ in these two cell lines. In contrast, after 30 min loading with caged S1P the cells became responsive to photolysis-mediated decaging. Increases in $[Ca^{2+}]_i$ in SKNMC cells were more prolonged than in HEK-293 cells (Fig. 3B), however, the cells were still viable because they still responded to

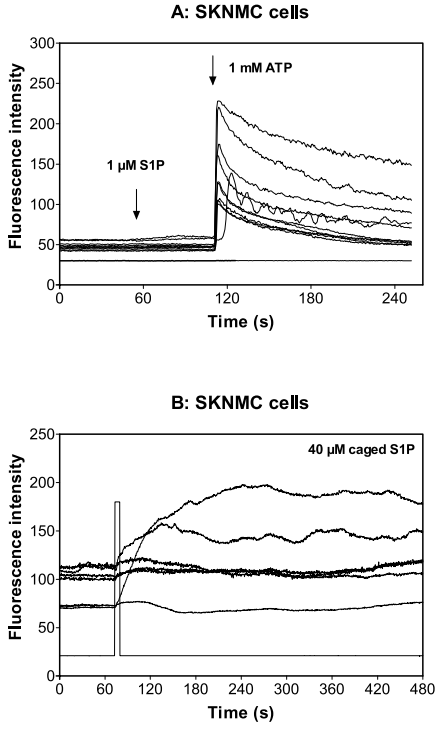

Fig. 3. $[Ca^{2+}]_i$ increase caused by intracellular but not extracellular S1P in SKNMC cells. A: Effects of 1 µM extracellular S1P and 1 mM ATP on $[Ca^{2+}]_i$, measured as fluo-4 fluorescence intensity, in cells of the human neuroepithelioma cell line SKNMC. B: Examples of $[Ca^{2+}]_i$ traces in six cells in response to photolysis of caged S1P. The cells were loaded for 60 min with caged S1P.

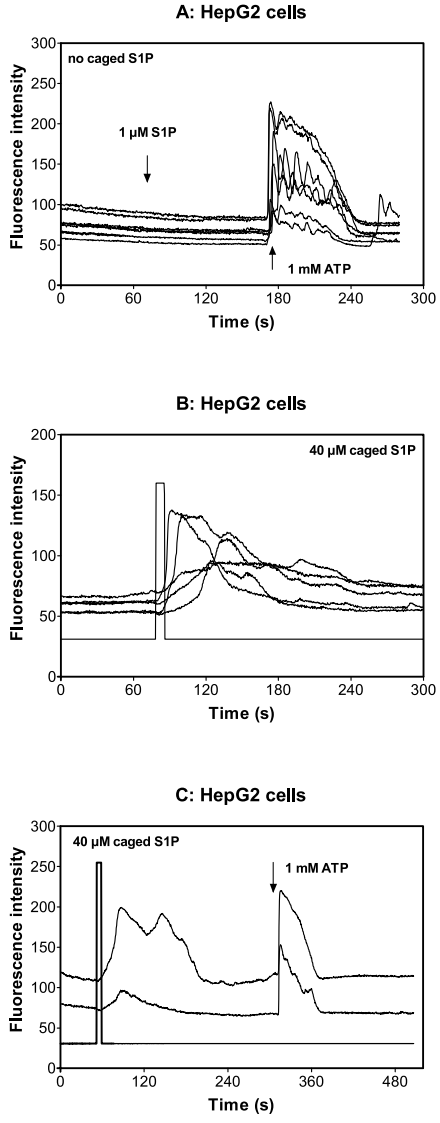

Fig. 4. $[Ca^{2+}]_i$ increase caused by intracellular but not extracellular S1P in HepG2 cells. A: Effects of 1 μM extracellular S1P and 1 mM ATP on $[Ca^{2+}]_i$, measured as fluo-4 fluorescence intensity, in cells of the human hepatocellular carcinoma cell line HepG2. B: Examples of $[Ca^{2+}]_i$ traces in five cells in response to photolysis of caged S1P. The cells were loaded for 70 min with caged S1P. C: Response of HepG2 cells to ATP addition after challenging with intracellular S1P. Two cells that had been loaded with caged S1P for 35 min were examined in this microscopic field.

the purinergic agonist (ATP; data not shown). The prolonged response observed in SKNMC cells might be due to differences in the inactivation of S1P by S1P lyase or phosphatases, or differences in extrusion of Ca^{2+} from the cytoplasm. In HepG2 cells, photolysis of caged S1P within seconds induced an elevation of $[Ca^{2+}]_i$ (Fig. 4B,C), which was transient, and sometimes oscillatory (Fig. 4C) and occasionally developed after a few seconds delay (Fig. 4B). Thus, the Ca^{2+} transients caused by intracellular S1P in HepG2 cells were very similar to those observed in PTX-treated HEK-293 cells (see Fig. 2). The Ca^{2+} stores were still functional after challenging with intracellular S1P, because a second release of S1P as well as agonist (ATP) addition after photolysis again caused an increase in $[Ca^{2+}]_i$ (Figs. 1D, 2B and 4C). In RH7777 cells, which also do not respond to extracellularly applied S1P with $[Ca^{2+}]_i$ transients [28] and for this reason are widely used for the heterologous expression of S1P-GPCR, photolysis of caged S1P also induced $[Ca^{2+}]_i$ increases. However, high 100–200 μM concentrations of caged S1P were required, making the other two cell lines a more suitable experimental model (data not shown).

Furthermore, in *Xenopus laevis* oocytes, extracellularly applied S1P up to 10 μM elicits no detectable Ca^{2+}-activated chloride current (I_{Cl}) [29]. Due to the large size of the oocytes (~1 mm diameter), microinjection of nanoliter volumes does not represent as big a perturbation as similar manipulations in cultured somatic cells. For this reason, we injected S1P into oocytes with the objective to obtain confirmation of S1P-elicited Ca^{2+} mobilization independently of the photolysis technique. Pressure microinjection of an estimated 1–10 nl of 0.5–2 mM S1P elicited the Ca^{2+}-dependent I_{Cl} with a mean amplitude of 17 ± 4 nA ($n = 12$), measured by standard two-electrode voltage clamp at a holding potential of −60 mV, while injection of the solvent alone had no effect.

Finally, in mammalian cells, we examined whether $[Ca^{2+}]_i$ transients induced by intracellularly applied S1P were caused by Ca^{2+} influx or by Ca^{2+} mobilization from intracellular stores. As shown in Fig. 5A, in a nominally Ca^{2+}-free buffer in the presence of 50 μM EGTA, photolysis of caged S1P elicited an increase in $[Ca^{2+}]_i$ in PTX-treated HEK-293 cells.

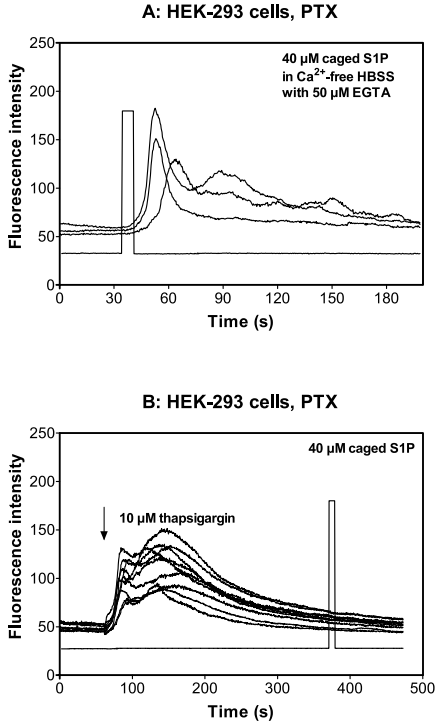

Fig. 5. Role of extracellular Ca^{2+} and thapsigargin-sensitive stores in $[Ca^{2+}]_i$ increase by intracellular S1P. A: Examples of $[Ca^{2+}]_i$ traces in three cells, measured as fluo-4 fluorescence intensity, in response to photolysis of caged S1P in the absence of extracellular Ca^{2+}. PTX-treated HEK-293 cells were loaded for 11 min with caged S1P in Ca^{2+}-free HBSS. Prior to the measurements, 50 μM EGTA was added. B: PTX-treated HEK-293 cells loaded for 42 min with caged S1P were first treated with 10 μM thapsigargin, followed 5 min later by photolysis of caged S1P. Nine cells were examined in this microscopic field.

Similar results were obtained in HepG2 cells kept in Ca^{2+}-free buffer (data not shown). These data indicate that in these two human cell types intracellular S1P elicits $[Ca^{2+}]_i$ transients by mobilizing Ca^{2+} from intracellular stores. This result does not exclude the possibility that in other cell types intracellular S1P may induce Ca^{2+} influx as shown recently in neutrophils [30]. The Ca^{2+} stores from which intracellular S1P releases Ca^{2+} were sensitive to thapsigargin, an inhibitor of the sarco-endoplasmic reticulum Ca^{2+} ATPase. Preincubation of PTX-treated HEK-293 cells for 5 min with 10 μM thapsigargin, which by itself caused an increase in $[Ca^{2+}]_i$, completely abolished the response to subsequent photolysis of caged S1P (Fig. 5B).

Taken together, we demonstrate that intracellular S1P, generated by photolysis of caged S1P, mobilizes Ca^{2+} in three human cell lines that otherwise do not mobilize Ca^{2+} in response to extracellularly applied S1P. Based on these data, we propose that S1P can mobilize Ca^{2+} independently of S1P-GPCR. Similarly to inositol-1,4,5-trisphosphate (IP_3) [31], S1P apparently mobilizes Ca^{2+} from thapsigargin-sensitive stores, most likely from the endoplasmic reticulum. This activity of S1P does not involve ryanodine receptors since they are not expressed in HEK-293 cells [32]. Likewise, SCaMPER, a protein suggested to be involved in sphingosylphosphorylcholine-mediated Ca^{2+} signalling [33], although this is controversial [34], appears not to play a role in Ca^{2+} mobilization by intracellular S1P. Using reverse transcriptase polymerase chain reaction, we could not detect SCaMPER mRNA in HEK-293 cells although this technique detected it in human cardiac myocytes (data not shown). An open question is whether the IP_3-gated Ca^{2+} channel is involved in the intracellular action of S1P. Ca^{2+} mobilization by microinjected S1P was not affected by heparin [16], but this does not exclude an allosteric action of S1P on the IP_3-gated Ca^{2+} channel. Since channel blockers, including xestospongin C and 2-aminoethoxydiphenyl borate, were not specific in HEK-293 cells (data not shown), this question will have to be further investigated in IP_3 receptor-deficient cells.

Until now, only indirect evidence had been provided for an intracellular second messenger role of S1P in Ca^{2+} signalling. Many different receptors, including the antigen receptors, growth factor receptors and several GPCR, stimulate sphingosine kinase activity or intracellular S1P production or both (for review see [35]) in a rapid and transient manner as expected for a process involved in Ca^{2+} mobilization [16–18,36–38]. Receptor-mediated Ca^{2+} mobilization was found to be sensitive to sphingosine kinase inhibitors [16–18,36,37] and to sphingosine kinase antisense oligonucleotides [19]. Although the exact target(s) mediating intracellular S1P-elicited Ca^{2+} mobilization has/have not been identified, the present findings are the first direct evidence that intracellular S1P mobilizes Ca^{2+} and reaffirm the hypothesis that sphingosine kinase-mediated S1P production is a receptor-controlled pathway for cellular Ca^{2+} signalling.

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