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Enhancement of intracellular sphingosine-1-phosphate production by inositol 1,4,5-trisphosphate-evoked calcium mobilisation in HEK-293 cells: endogenous sphingosine-1-phosphate as a modulator of the calcium response

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Abstract

Sphingosine-1-phosphate (S1P) regulates many cellular functions, such as migration, differentiation and growth. The effects of S1P are thought to be primarily mediated by G-protein coupled receptors, but an intracellular function as a calcium releasing second messenger has also been proposed. Here we show that in HEK-293 cells, exogenous S1P mobilises sequestered calcium by a mechanism primarily dependent on the phospholipase C (PLC)/inositol 1,4,5-trisphosphate (IP_3) pathway, and secondarily on the subsequent synthesis of intracellular S1P. Stimulating HEK-293 cells exogenously with S1P increased the production of both inositol phosphates and intracellular S1P. The calcium response was inhibited in cells treated with 2-APB, caffeine or U73122, showing that the PLC/IP_3 pathway for calcium release is activated in response to exogenous S1P. The calcium response was partially inhibited in cells treated with the sphingosine kinase inhibitor DMS and in cells expressing a catalytically inactive sphingosine kinase, showing that endogenously produced S1P is also involved. Importantly, 2-APB and U73122 inhibited the S1P-evoked production of intracellular S1P. S1P is therefore not likely a major calcium releasing second messenger in HEK-293 cells, but rather a secondary regulator of calcium mobilisation.

Keywords: Inositol 1,4,5-trisphosphate; Sphingosine kinase; Calcium; Second messenger; Phospholipase C; Receptor

1. Introduction

Derivatives of sphingomyelin take part in many signaling events. Elevated levels of ceramide and sphingosine have been associated with apoptosis [1,2], while sphingosine-1-phosphate (S1P) stimulates cell survival and growth, as well as regulates migration and differentiation [3–6].

Formation of S1P from sphingosine and ATP is catalyzed by sphingosine kinase. Two isoforms of sphingosine kinase have been cloned [7–9]. The function and regulation of type 1 sphingosine kinase (SK1) has been an object of thorough investigations. It has been shown that SK1 is translocated to the cell membrane in a Ca^{2+}-calmodulin dependent fashion [10] and that its activation is dependent on protein kinase C (PKC) [11] and ERK1/2 [12]. SK1 may also be exported and produce S1P extracellularly, but in contrast to intracellular SK activity, the extracellular activity

Abbreviations: S1P, sphingosine-1-phosphate; SK, sphingosine kinase; PKC, protein kinase C; IP_3, inositol 1,4,5-trisphosphate; 2-APB, 2-aminoethyl diphenyl borate; IP_x, inositol phosphates; HBSS, HEPES buffered salt solution; cS1P, caged sphingosine-1-phosphate; PLC, phospholipase C; DMS, D-*erythro*-*N*,*N*-dimethylsphingosine.

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was not stimulated by growth factors or PKC activation [13].

S1P initially raised much interest as a calcium mobilizing second messenger [14,15]. Later it was shown that S1P is a high affinity ligand for a family of G-protein coupled receptors [16,17]. Five of these receptors have to date been identified and are named $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$ and $S1P_5$ (formerly EDG-receptors). Since it is generally assumed that S1P has both intra- and extracellular sites of action, there is some confusion in the literature regarding how S1P regulates different cellular functions. There are reports suggesting that both extra- and intracellular S1P can affect proliferation [18–21], as well as migration [22–24]. An intracellular target for S1P has not been identified, but several investigations have shown a direct calcium mobilizing effect of intracellular S1P [14,15,25,26]. One report also suggests that intracellular S1P is a calcium influx factor, mediating store operated calcium entry [27]. As cellular SK activity can be stimulated by increased levels of intracellular calcium, it is reasonable to assume that a calcium trigger stimulates S1P production, resulting in S1P-induced release of sequestered calcium [28–30]. However, the activation of SK and subsequent S1P mediated calcium mobilisation has in some studies been shown to occur independently of other calcium mobilizing second messengers [31–33]. One such system where S1P synthesis, but not IP_3 production, could be detected previous to calcium mobilisation was when HEK-293 cells were stimulated with extracellular S1P [33]. In the work presented here we have re-examined the mechanism for calcium release mediated through G-protein coupled S1P receptors in HEK-293 cells. We report three main findings: 1) IP_3 is the major calcium releasing second messenger in this system. 2) The agonist-induced enhancement of intracellular S1P production is dependent on calcium release from IP_3 receptors. 3) Endogenously produced S1P modulates the calcium response.

2. Materials and methods

2.1. Materials

Fura-2/AM and DMNP-EDTA/AM were from Molecular Probes (Eugene, OR, USA). Amprep trimethylaminopropyl SAX mini columns and myo-[^{3}H] inositol were from Amersham Biosciences (Little Chalfont, Bucks., UK). D-*erythro*-Sphingosine-1-phosphate and D-*erythro*-*N*,*N*-dimethylsphingosine were from Biomol (Plymouth Meeting, PA, USA). Minimum essential medium (MEM with Earle's salts, without L-glutamine) and L-glutamine were from GIBCO (Grand Island, NY, USA). Cell-Tak™ was from BD Biosciences (Bedford, MA, USA). Perchloric acid was from Merck (Darmstadt, Germany). Caffeine, 2-APB, $GdCl_3$, *N*-acetyl-D-sphingosine, ATP (sodium salt), bovine serum albumin (BSA, essentially fatty acid free), poly-D-lysine, tri-*n*-octylamine, D-*erythro*-sphingosine, the monoclonal anti-FLAG M2 antibody and 1,1,2-trichlorotrifluoroethane were from Sigma (St. Louis, MO, USA). [^{3}H] Sphingosine was from NEN Life Science Products (Boston, MA, USA). Caged sphingosine-1-phosphate (cS1P) was from Alexis Corporation (San Diego, CA, USA).

2.2. Cell culture and transfection

HEK-293 cells were cultured in MEM, containing 2 mM L-glutamine, 6% fetal calf serum, 50 U/ml penicillin and 50 μg/ml streptomycin. Cells were grown in a humidified incubator with 5% CO_2 and 95% air at 37 °C. Transient transfection was performed using the calcium phosphate precipitation method [34] with 2 μg of empty pcDNA3 vector, pcDNA3-hSK^{WT}(FLAG) or pcDNA3-hSK^{G82D}(FLAG) [35] per 35 mm petri dish together with 1 μg of EYFP for identification of transfected cells.

2.3. Calcium measurements

Cells cultured on poly-D-lysine coated coverslips were washed twice with HEPES-buffered salt solution (HBSS) consisting of 118 mM NaCl, 4.6 mM KCl, 1 mM $CaCl_2$, 10 mM glucose and 20 mM HEPES (pH 7.4) and incubated with 2 μM Fura-2/AM for 30 min at 37 °C. The cells were then washed with HBSS and incubated for 15 min at room temperature. The coverslip was placed in a perfusion chamber that was mounted on a Zeiss Axiovert 35 microscope. The 340 nm and 380 nm excitation filters were used and emission was measured at 510 nm. Light was obtained from an XBO 75W/2 xenon lamp. The shutter was controlled by a Lambda 10-2 control device (Sutter Instruments, Novato, CA, USA), and images were collected with a SensiCam CCD camera (PCO/CD Imaging, Kelheim, Germany). The images were aquired every 2.5 s, and were processed using Axon Imaging Workbench 2.1 software (Axon Instruments, Foster City, CA, USA). The experiments were performed at room temperature. The system was calibrated in situ, and the program Axon Imaging Workbench automatically calculated $[Ca^{2+}]_i$ according to Grynkiewics et al. [36].

In the experiments performed on transiently transfected cells, the results are given as the percentual increase in $[Ca^{2+}]_i$ compared to non-transfected cells on the same coverslip. This was done to reduce the effect of variability between coverslips.

For calcium uncaging experiments, cells were prepared as above, except that 4 μM DMNP-EDTA/AM was added along with Fura-2/AM. The experiments were performed with cells in HBSS with high calcium (10 mM) to raise $[Ca^{2+}]_i$ and allow for more effective uncaging.

For experiments on permeabilised cells, HEK-293 cells were plated on Cell Tak coated cover slips the day before an experiment. Cells were loaded with 3 μM mag-Fura-2/AM for 30 min at 37 °C and then washed twice with KCl rinse buffer (125 mM KCl, 25 mM NaCl, 10 mM HEPES and 0.2

mM $MgCl_2$, pH 7.25). Cells were then permeabilized with intracellular buffer (KCl rinse buffer+3 mM ATP+200 μM $CaCl_2$+500 μM EGTA, pH 7.25) containing 10 μg/ml digitonin. The permeabilisation process was monitored continuously. When approximately 80% of the cells were permeabilized, excess digitonin was rinsed away by perfusion with intracellular buffer. Before every experiment pH was checked to be 7.25 in all solutions used to stimulate permeabilized cells.

The effects of the inhibitors on basal $[Ca^{2+}]_i$ was determined using a Hitachi F-2000 Fluorescence spectrophotometer. The method was as described previously [37].

2.4. Uncaging of sphingosine-1-phosphate

Cells were treated with 100 ng/ml pertussis toxin over night to block calcium responses mediated through receptors in the plasma membrane. Cells were loaded with Fura-2/AM, washed with HBSS, and incubated for 20 min with 10 μM cSPP. The coverslips were then mounted in a perfusion chamber. To prevent leakage of cSPP out of the cells, 10 μM cSPP was added to the medium. Uncaging started immediately with the $[Ca^{2+}]_i$ measurements.

2.5. Measurements of inositol phosphates

Cells were plated onto 100 mm dishes and cultured for 24 h. Medium containing 1 μCi/ml of myo-[^{3}H] inositol was added to the cells 48 h prior to an experiment. Cells were suspended and washed three times in HBSS and incubated at 37 °C for 10 min. The cells were then pelleted by centrifugation and the buffer was replaced with HBSS containing 10 mM LiCl. Cell suspension (500 μl) was incubated with agonist or vehicle for 10 min. The reactions were stopped with 500 μl ice cold 10% perchloric acid. The mixtures were incubated 10 min on ice and centrifuged at 1000×*g* for 5 min. 600 μl of the supernatant was transferred to a test tube with 150 μl 10 mM EDTA (pH 7.0). 450 μl of a 1,1,2-trichlorotrifluoroethane/tri-*n*-octylamine mixture (1:1, v/v) was added and the tubes were vortex-mixed for 1 min. Phases were separated by centrifugation (1 min, 1000×*g*). Neutralized sample (400 μl) was transferred to a new test tube. The sample was then transferred to an Amprep SAX mini column that had been washed with 5 ml 1 M $KHCO_3$ and 10 ml H_2O. The solution was passed through the column and was subsequently washed with 5 ml of H_2O. IP_x was eluted with 5 ml of 0.17 M $KHCO_3$. One milliliter of the eluted material was transferred to scintillation tubes and 4 ml scintillation fluid was added. The contents were mixed for 1 h before measurement.

2.6. Measurement of sphingosine-1-phosphate production

Cells grown on 35 mm petri dishes were incubated over night in medium with serum replaced by 0.2% fatty acid free BSA. Cells were then equilibrated in 800 μl MEM+25 mM HEPES+0.1% fatty acid free BSA (pH 7.4) for 10 min at 37 °C. After incubation with inhibitor or vehicle, medium+[^{3}H] sphingosine (~200,000 cpm) with or without S1P (final concentration 2 μM) was then added. After incubating at 37 °C for 30 s, the medium was rapidly aspirated and the reaction stopped with 500 μl of ice-cold methanol. The cells were scraped and transferred to polypropylene tubes. The dishes were extracted once again with 500 μl methanol. The tubes were sonicated twice for 20 s each and centrifuged at 6000×*g* for 10 min to remove cell debris. The supernatant was then transferred to glass vials. D-*erythro*-Sphingosine and S1P were added to each sample for identification and the supernatant was evaporated. After redissolving in methanol the samples were spotted onto HPTLC plates and separated with butan-1-ol/acetic acid/water (3:1:1, v/v). Sphingosine and S1P were stained with ninhydrin and spots were scraped into vials for liquid scintillation and the radioactivity was measured.

2.7. Immunocytochemistry

Cells were fixed in ice-cold methanol for 6 min and were then washed three times with PBS. Cells were incubated with anti-FLAG M2 in TBS+2% milk powder (1:1000) for 45 min at 37 °C, washed and labelled with secondary antibody (Alexa 488 goat anti-mouse, 1:500) in TBS+2% milk powder for another 45 min at 37 °C. The coverslips were then washed in PBS and mounted on an object glass using a 4:1 mix of PBS/glycerol. For the translocation experiments, cells were challenged with either 10% FCS, 2 μM S1P or vehicle for 5 min. The cells were then fixed and stained as above and analysed using confocal microscopy. Six sections ~0.25 μm apart were scanned in the middle of the cell. The data was quantified as the ratio of fluorescence at the plasma membrane and the average cytoplasmic fluorescence in the max projection of the scanned sections.

2.8. Western blotting

48 h post transfection cells from 100 mm petri dishes were washed once with cold HBSS, and scraped in 200 μl lysis buffer (10 mM Tris/HCl (pH 7.7), 150 mM NaCl, 7 mM EDTA, 0.5% NP-40, 0.2 mM PMSF and 0.5 μg/ml leupeptin). Lysates were kept on ice for 15 min and were then centrifuged at 10,000×*g* for 15 min. 100 μl 3× Laemmli's buffer was mixed with the supernatant and the solution was heated to 95 °C for 3 min. Proteins were separated by 10% SDS–PAGE and blotted onto a nitrocellulose membrane. For detection of the FLAG tagged sphingosine kinases the monoclonal anti-FLAG M2 was used as primary antibody (1:1000) and HRP conjugated rabbit anti mouse as secondary antibody (1:1000). Bands were visualised by dipping the nitrocellulose membrane in a DAB–substrate solution (0.1 M Tris/HCl (pH 7.3), 0.8 mg/ml DAB and 0.1‰ H_2O_2). Membranes were then washed in MQ-H_2O and dried.

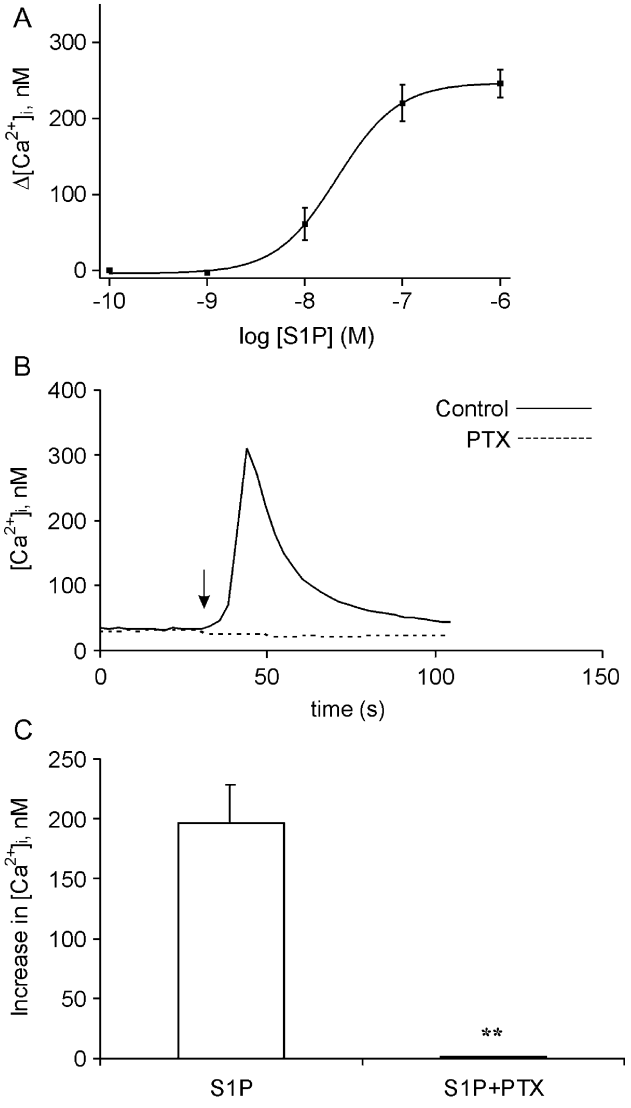

Fig. 1. Effect of S1P on $[Ca^{2+}]_i$ in HEK-293 cells. Cells in monolayer were incubated with Fura-2 as described in the *Experimental Procedures*. (A) Concentration dependence of the S1P evoked Ca^{2+} response measured on cells in monolayer. The values given are the mean±S.E.M. of measurements made in 5–17 cells. (B) Representative traces showing the response to stimulation with 2 μM exogenous S1P in a single control cell and a cell incubated for 16 h with 100 ng/ml pertussis toxin. (C) Summary graph showing the sizes of the calcium responses to 2 μM S1P in control cells and in pertussis toxin treated cells (n=19, $^{**}p<0.01$).

2.9. Statistics

Results are expressed as means±S.E.M. Statistical analysis was made using Student's t test for paired observations. When three or more means were tested, one-way ANOVA was performed followed by Bonferroni's multiple comparisons test or Dunnett's test for multiple comparisons against a single control. For data containing three or more groups that did not follow normal distribution, the Kruskal–Wallis statistic was used followed by Dunn's test. Curve fitting was done using the Prism program.

3. Results and discussion

3.1. Effect of exogenous S1P on $[Ca^{2+}]_i$

Stimulating HEK-293 cells with S1P induced a transient and concentration-dependent increase in $[Ca^{2+}]_i$ (Fig. 1).

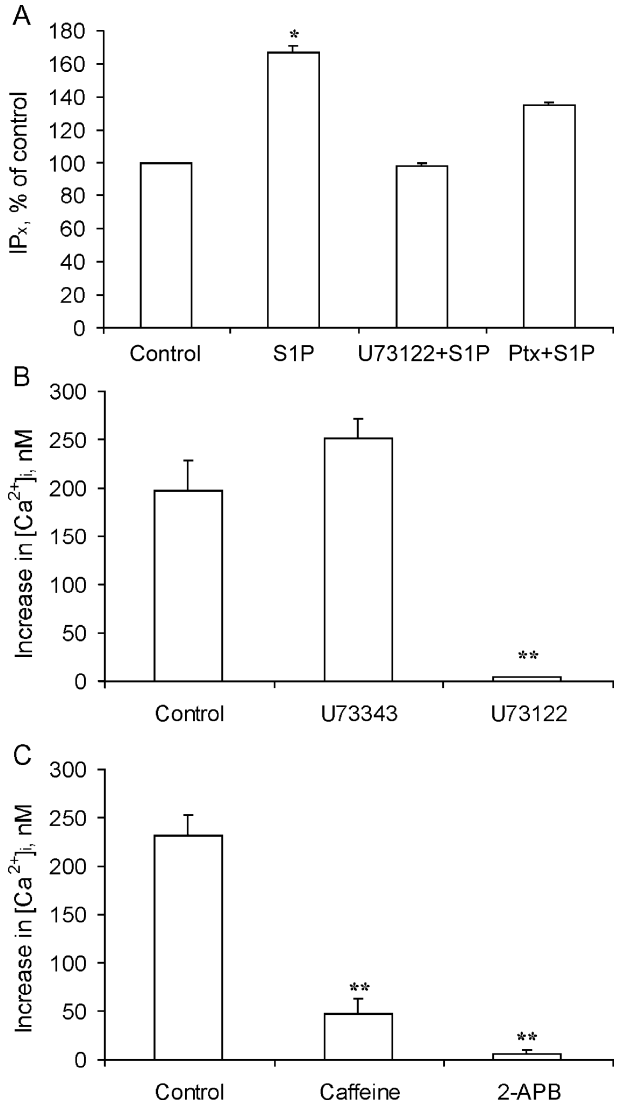

Fig. 2. Exogenous S1P mobilises calcium through the PLC/IP_3 pathway. (A) *Effect of exogenous S1P on inositol phosphate formation.* Cells were incubated with 1 μCi/ml of myo [^{3}H] inositol for 48 h. Cells were treated with 100 ng/ml pertussis toxin for 16 h, 10 μM U73122 or vehicle for 2 min and were then stimulated with 2 μM S1P or vehicle. The bars give the mean±S.E.M. of three separate determinations. (B) *U73122 inhibits the S1P induced increase in $[Ca^{2+}]_i$.* Cells grown on coverslips were loaded with Fura-2 as described in the *Experimental Procedures*. After incubating for 3 min with 10 μM of either U73122 or U73343, or with vehicle, the cells were stimulated with 2 μM S1P. The bars give the mean±S.E.M. of at least 18 cells measured in each group. (C) *Effect of the IP_3R inhibitors 2-APB and caffeine on the S1P evoked calcium response*. Cells grown on coverslips were loaded with Fura-2 and incubated with 20 mM caffeine or 50 μM 2-APB for 2 min prior to stimulation with 2 μM S1P. The bars give the mean±S.E.M. of at least 46 measured cells in each group. ($^*p<0.05$; $^{**}p<0.01$).

Preincubating the cells with pertussis toxin (100 ng/ml) for 16–24 h attenuated the Ca^{2+} response indicating that the βγ-dimer of a $G_{i/o}$ protein mediates the response [38,39].

3.2. S1P receptors activate the PLC/IP_3 pathway for calcium release

Next, we investigated by which mechanisms exogenous S1P evokes the Ca^{2+} signal. It has previously been proposed that IP_3 is not involved in the calcium mobilisation evoked by exogenous S1P in HEK-293 cells [33]. We re-examined this claim after finding in preliminary experiments that the IP_3 receptor inhibitor 2-APB effectively inhibited the calcium mobilisation. First, total accumulated inositol phosphates (IP_x) were measured after stimulating the cells with 2 μM S1P or vehicle for 10 min. IP_x was increased by 67% compared with control cells after stimulation with S1P. In cells pre-treated with the PLC-inhibitor U73122 the S1P-induced IP_x production was completely inhibited. Interestingly, in pertussis toxin treated cells the inhibition was only ~50% (Fig. 2A), even though the calcium response was abolished in these cells (see Fig. 1C).

To further establish whether the PLC/IP_3 pathway is involved, we tested the effect of PLC inhibition on the S1P evoked calcium response. Pretreating the cells for 3 min with 10 μM of the PLC-inhibitor U73122 effectively blocked the S1P evoked calcium response, whereas the inactive analogue U73343 was without an effect (Fig. 2B). The basal $[Ca^{2+}]_i$ levels after the treatments were: Control cells 114±7 nM, U73343-treated cells 122±3 nM and for U73122 treated cells 143±8 nM. The IP_3 receptor inhibitors 2-APB and caffeine [40,41] were also effective in inhibiting the calcium response following stimulation with exogenous S1P. The calcium mobilisation was almost abolished after pre-treatment with 2-APB (50 μM) or caffeine (20 mM) (Fig. 2C). In these cells the basal $[Ca^{2+}]_i$ level was 137±19 nM in control cells, 98±10 nM in 2-APB-treated cells and 159±13 nM in caffeine-treated cells. 2-APB has been shown to be a somewhat unreliable inhibitor of IP_3 receptors, since it effectively blocks store operated calcium channels in the plasma membrane, and may also inhibit SERCA pumps (see [42]). In our experiments the effect of 2-APB was not due to inhibition of store operated channels, since an inhibition of the calcium response was observed both in calcium containing and calcium free buffer. Furthermore, 2-APB at the concentration used did not increase the calcium leak from intracellular stores, nor did it empty thapsigargin sensitive stores (results not shown). Having established that the PLC/IP_3 pathway is crucial for the calcium release evoked by exogenous S1P, we addressed the proposed role of intracellular S1P as a calcium-mobilizing second messenger in this system [33].

3.3. The response evoked by exogenous S1P is dependent on multiple calcium-releasing mechanisms

We investigated whether the calcium-response evoked by exogenous S1P is dependent on other calcium-releasing

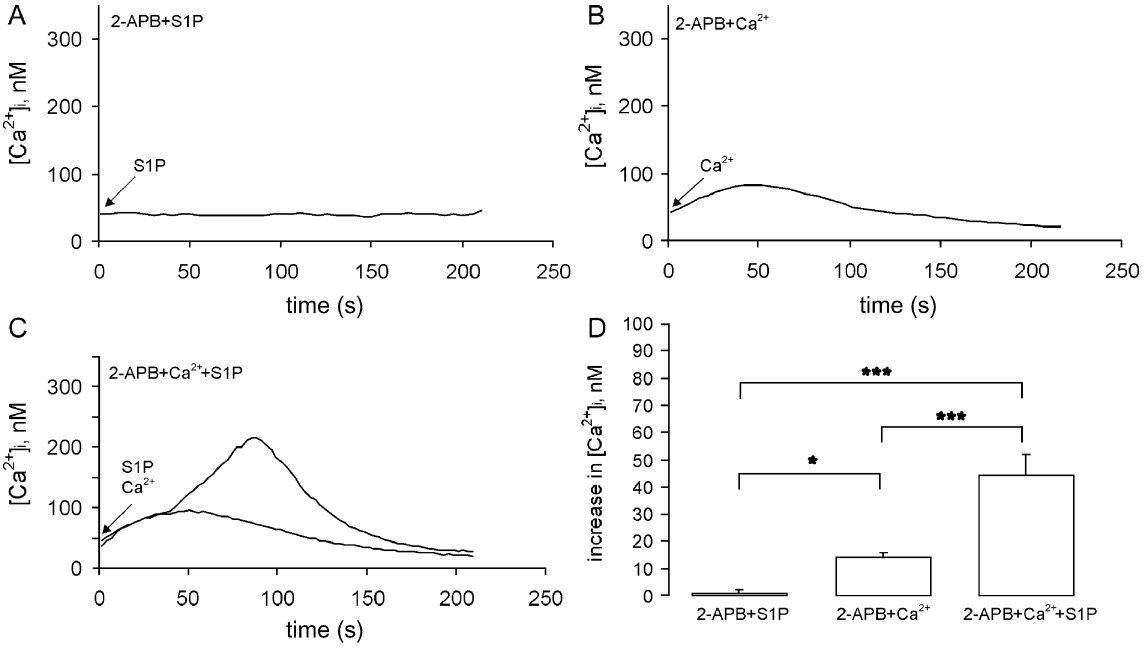

Fig. 3. Artificially raising $[Ca^{2+}]_i$ partially restores the S1P-evoked calcium response in 2-APB treated cells. Cells cultured on coverslips were loaded with Fura-2. The traces shown are representative of at least 17 cells measured in each group. (A) Cells were incubated for 2 min with 50 μM 2-APB and were challenged with 2 μM exogenous S1P at the start of the Ca^{2+} measurement. (B) Cells loaded with the calcium cage DMNP-EDTA (4 μM for 30 min) were incubated for 2 min with 50 μM 2-APB. Uncaging of Ca^{2+} was started immediately with the measurement. (C) Cells were treated as in B, except that the cells were stimulated with 2 μM S1P at the beginning of the measurement. (D) Summary of the calcium responses obtained in A, B and C. $\Delta[Ca^{2+}]_i$ was calculated as the difference between the maximum $[Ca^{2+}]_i$ and the $[Ca^{2+}]_i$ of the first frame. The bars give the mean±S.E.M. (*p<0.05; ***p<0.001).

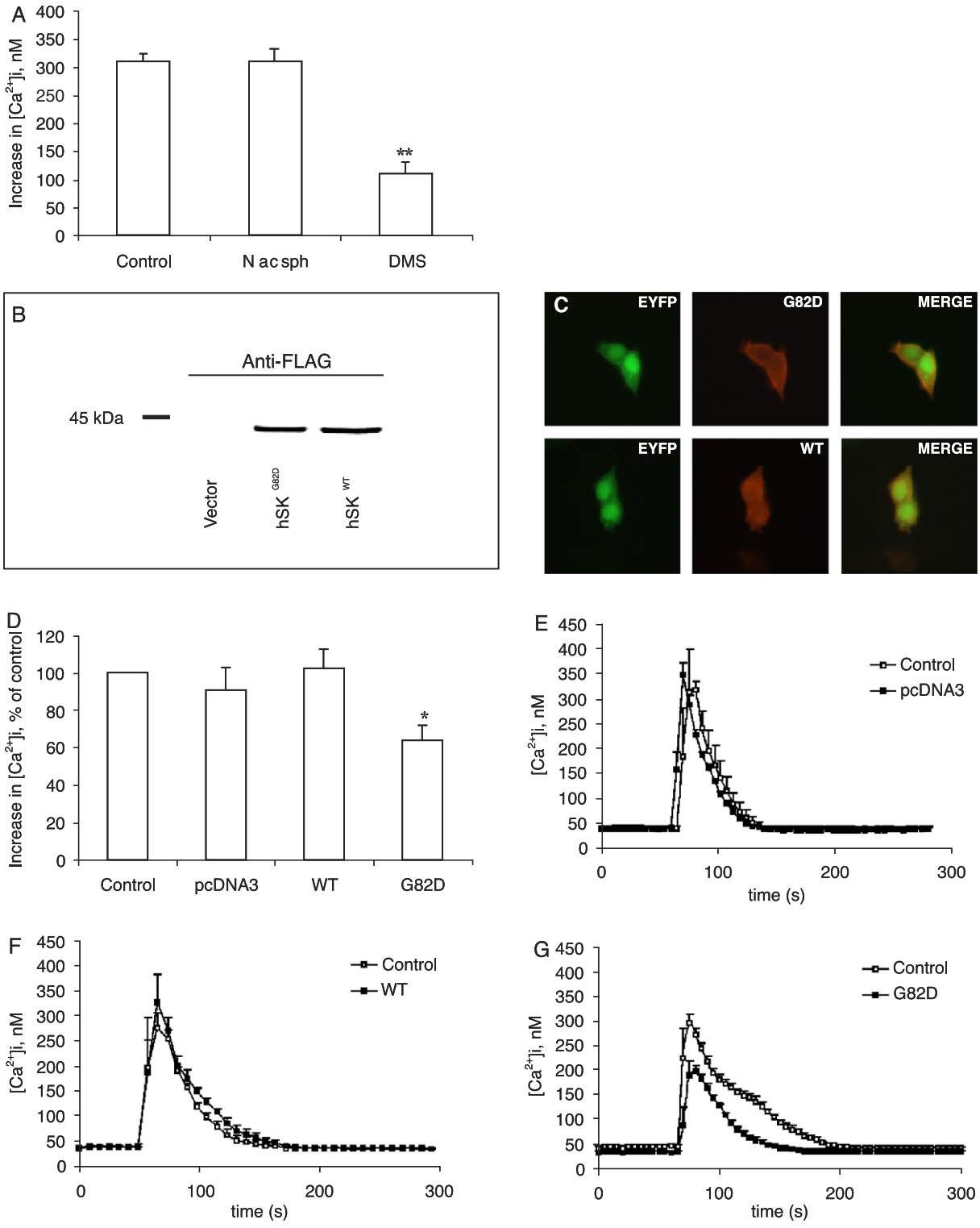

Fig. 4. Inhibitory effect of DMS and kinase dead SK^{G82D} on the S1P-mediated calcium mobilisation. (A) Cells grown on coverslips were loaded with Fura-2, incubated 2–4 min with 10 μM DMS, 30 μM *N*-acetyl-D-sphingosine or vehicle and then stimulated with 2 μM S1P. The bars give the mean±S.E.M. of at least 29 cells measured in each group (**$p<0.01$). (B) HEK-293 cells transfected with either vector (pcDNA3), hSK^{G82D} or hSK^{WT} were lysed 2 days post transfection and analysed by Western blotting. In the lysates from hSK transfected cells the mouse anti-FLAG M2 primary antibody detected a band of ~45 kDa. (C) HEK-293 cells were cotransfected with SK and EYFP for identification. (D) Cells transfected with either empty vector, SK^{WT} or the catalytically inactive SK^{G82D} were stimulated with 2 μM S1P in Ca^{2+}-free buffer. The bars give the percentual increase in $[Ca^{2+}]_i$ ±S.E.M. compared to non-transfected cells on the same coverslip (n=19 for pcDNA3, n=30 for SK^{WT} and n=31 for SK^{G82D}) (*$p<0.05$). (E–G) Representative traces of the data shown in (D). The graphs show the response evoked by 2 μM S1P in non-transfected control cells and transfected cells on a single coverslip. Each trace shows the average response ±S.E.M. of four measured cells.

agents in addition to IP_3. When stimulating 2-APB treated cells with S1P there was a response in only 1 out of 24 measured cells, indicating that the calcium mobilisation is primarily mediated by IP_3 (Fig. 3A,D). However, the response could be partially restored in 2-APB treated cells by intracellularly uncaging calcium simultaneously to application of exogenous S1P. There was a distinct response in 5 out of 32 measured cells treated this way, but more importantly, the overall increase in $[Ca^{2+}]_i$ was significantly higher than in the control cells where no S1P was applied (Fig. 3B,C,D). These results show that the calcium response evoked by exogenous S1P consists of an initial 2-APB sensitive calcium mobilisation followed by a calcium dependent and 2-APB insensitive mobilisation. Since it has been shown that an elevation in $[Ca^{2+}]_i$ is needed for activation [29] and translocation [10] of sphingosine kinase to the plasma membrane, we tested whether sphingosine kinase could be responsible for the calcium mobilisation following IP_3 mediated calcium release.

3.4. The calcium mobilisation induced by exogenous S1P is partially dependent on endogenous S1P production

Preincubating HEK-293 cells with the sphingosine kinase inhibitor *N*,*N*-dimethylsphingosine (DMS) significantly diminished the calcium response elicited by exogenous S1P, whereas pretreating cells with the structurally similar lipid *N*-acetyl-D-sphingosine had no noticeable effect (Fig. 4A). To get a more specific inhibition of sphingosine kinase, we used cells transiently transfected with either the wild type SK or the kinase dead SK^{G82D} [35]. A western blot revealed strong expression of ~45 kDa proteins in cells transfected with pcDNA3-hSK^{WT}(FLAG) and pcDNA3-hSK^{G82D}(FLAG) but not in cells transfected with empty pcDNA3 vector (Fig 4B). Initial tests also confirmed that the SK^{WT} was functional and increased cellular S1P by approximately 400%. Briefly, transfected cell cultures and control cells were incubated with 200,000 cpm [^{3}H] sphingosine for 15 min, and the cellular [^{3}H] S1P was measured according to the procedure in the Materials and Methods (results not shown). Cells were transfected with either hSK^{WT} or hSK^{G82D} together with EYFP for identification (Fig. 4C) and their calcium responses to exogenous S1P were compared. To correct for variations between coverslips in response sizes, transfected cells were normalized against the responses of non-transfected cells on the same coverslip. Cells transfected with the kinase dead SK^{G82D} exhibited a significantly smaller calcium transient than cells transfected with the wild type SK (Fig. 4D–G), suggesting that intracellular S1P indeed affects calcium signaling in these cells.

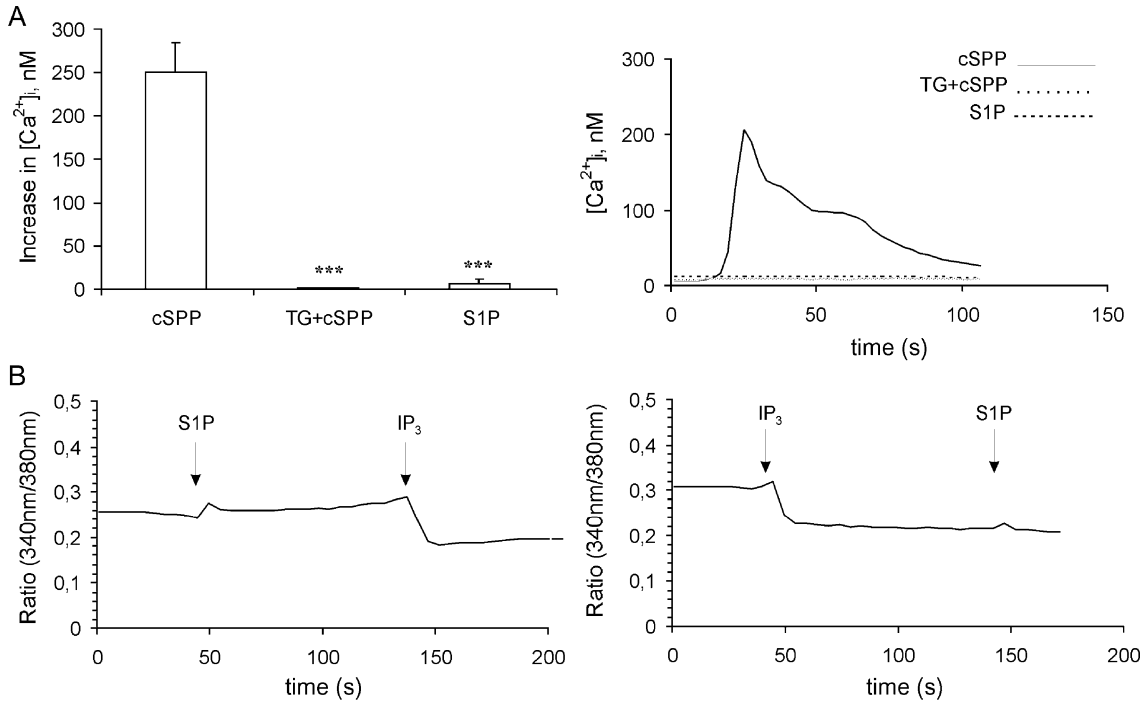

Fig. 5. Intracellular S1P evokes an increase in $[Ca^{2+}]_i$. (A) Cells treated with pertussis toxin (100 ng/ml, overnight) were loaded with Fura-2 and caged S1P or vehicle as described in the *Experimental Procedures*. S1P was uncaged in cS1P-loaded cells (cSPP) or in cS1P-loaded cells that had been pretreated with 1 μM thapsigargin for 5 min (TG+cSPP). As a control, pertussis toxin-treated cells were stimulated with 10 μM exogenous S1P to show that the response mediated through GPCRs was inactivated (S1P). The bars give the mean response ±S.E.M. of at least 20 cells measured in each group (***$p<0.001$). The graph to the right shows representative traces of calcium responses when uncaging S1P and exogenously adding 10 μM S1P to pertussis toxin treated cells. (B) S1P alone does not release Ca^{2+} from IP_3-sensitive stores in permeabilised HEK-293 cells. HEK-293 Cells were loaded with the low affinity Ca^{2+} indicator mag Fura-2, and permeabilised as described in the *Experimental Procedures*. Cells were then challenged with 5 μM IP_3 or 5 μM S1P. The traces are representative of 11 cells measured where S1P was added first, and 15 cells where IP_3 was added first.

Since inhibition of SK reduced the Ca^{2+} response, we investigated whether uncaging S1P intracellularly would mobilise calcium. In the following series of experiments cells were treated with 100 ng/ml pertussis toxin overnight to inhibit calcium mobilisation through G-protein coupled receptors. Uncaging of S1P in pertussis toxin treated cells induced a mobilisation of calcium, whereas there was no calcium response when stimulating the cells with 10 μM exogenous S1P (Fig. 5A). Intracellular uncaging of S1P stimulated calcium release from thapsigargin-sensitive stores, since the calcium mobilisation was abolished in cells that were pre-treated with 1 μM thapsigargin for 5 min (Fig. 5A). Perfusing the cells with 10 μM sphingosine or 10 μM C2-ceramide did not evoke a calcium response (results not shown). These results suggest that the effect of S1P is in this case intracellular and not due to export of S1P or its metabolites.

We then investigated whether intracellular S1P mobilizes calcium by directly acting on an intracellular Ca^{2+} releasing channel. Cells were loaded with the low affinity calcium probe mag-Fura-2, permeabilized, and then challenged with S1P or IP_3. S1P failed to release calcium, whereas IP_3 effectively did so (Fig. 5B). We did not observe any synergistic effects between S1P and the IP_3-mediated calcium release. These results suggest that the calcium-mobilising effect of intracellular S1P is indirect, and not due to direct activation of a calcium release channel. Alternatively, S1P sensitive calcium stores may have been disrupted by the permeabilization process.

3.5. S1P synthesis is enhanced by IP_3 mediated Ca^{2+} release

Next, we investigated how the PLC/IP_3 pathway and intracellular S1P production relate to each other. We detected a modest increase in intracellular S1P-synthesis 30 s after stimulation with exogenous S1P (134±7% compared with unstimulated control cells $p<0.05$). The increase in S1P was attenuated in cells that were pre-treated with 2-APB, U73122 or pertussis toxin, showing that the IP_3 mediated calcium release is necessary for enhancing the synthesis of endogenous S1P (Fig. 6A). However, this does not rule out the possibility that other extracellular agonists may stimulate S1P synthesis by a mechanism that is independent of previous calcium mobilisation. We therefore tested whether the S1P production induced by application of 10% fetal calf serum (FCS) to serum-starved cells was similarly attenuated by Ptx, 2-APB and U73122 (Fig. 6B). FCS contains several growth factors, hormones and bio-active lipids and therefore activates multiple signaling pathways, and also strongly activates SK. The increase in intracellular S1P following FCS stimulation was also in this case attenuated by inhibitors of the PLC/IP_3 pathway for calcium release. It is therefore unlikely that S1P is a primary calcium releasing second messenger in HEK-293 cells, but instead

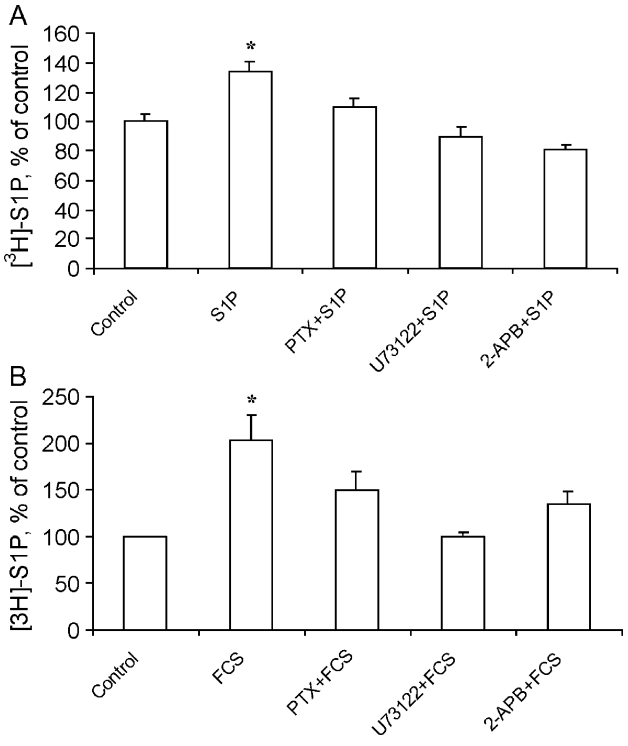

Fig. 6. SK-activation is dependent on IP_3R mediated calcium release. (A) Cells were incubated with 100 ng/ml pertussis toxin for 16 h, 50 μM 2-APB, 10 μM U73122 or vehicle for 2 min, and were then stimulated with 2 μM S1P for 30 s. The data are expressed as the percent of formed [^{3}H] S1P compared with unstimulated control cells. The bars give the mean±S.E.M. ($n>6$). (B) Same as in A, but the cells were stimulated with 10% FCS instead of S1P ($n=3$).

functions as a modulator of the IP_3-mediated response. In subsequent experiments we found that pretreating the cells with the MAP-kinase kinase inhibitor PD98059 (50 μM for 20 min) or the protein kinase C inhibitor Calphostin C (100 nM for 2 min) attenuated the S1P mediated increase in S1P synthesis, (93±16% and 114±12%, respectively, compared with unstimulated control cells). This is in line with earlier reports showing PKC and ERK dependent activation of SK [11,12].

We also tested whether the enhanced S1P production was accompanied by translocation of SK to the plasma membrane. HEK-293 cells transfected with SK^{WT} were serum-starved over night and then stimulated with either 10% fetal calf serum, 2 μM S1P or vehicle for 5 min prior to fixation and immunocytochemical staining of SK. We did not observe a statistically significant translocation of SK during this time span (Fig. 7). In an attempt to address calcium dependent translocation of SK, cells were stimulated with 1 μM of thapsigargin for 10 min prior to fixation. Neither in this case did we observe a statistically significant translocation (results not shown), even though it has been shown to take place [10]. It has to be mentioned that there was a very high occurrence of cytoplasmic SK in over-expressing cells, which may mask translocation to the plasma membrane.

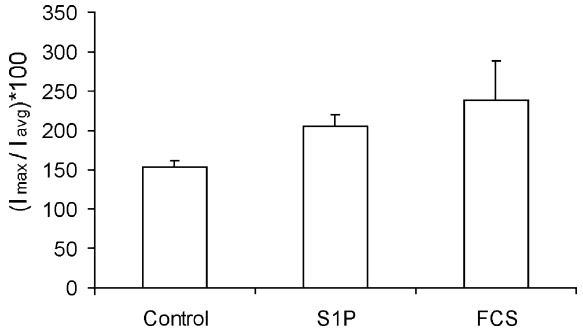

Fig. 7. Agonist effect on SK translocation. Cells overexpressing SK were stimulated with 10% FCS, 2 μM S1P or vehicle for 5 min prior to fixing and staining for SK. The cells were analysed by confocal microscopy and the data is presented as the percentual fluorescence intensity in the vicinity of the plasma membrane compared to the average cytoplasmic fluorescence intensity. The bars give the mean±S.E.M. of 10 determinations.

In conclusion, we have shown that stimulating HEK-293 cells with exogenous S1P elicits a calcium response that is dependent primarily on IP_3. This calcium mobilisation enhances the production of cellular S1P which modulates the calcium response. The calcium response was completely abolished in cells treated with the PLC-inhibitor U73122 or the IP_3R inhibitor 2-APB, showing that IP_3 is the primary calcium mobilising second messenger in this signaling pathway. Pertussis toxin, 2-APB and U73122 also inhibited the agonist-induced S1P production, suggesting that it is dependent on an initial IP_3-mediated calcium mobilisation. Many studies concerning S1P as a calcium releasing second messenger have relied on somewhat non-specific pharmacological agents to inhibit SK, making the subject a matter of much debate. For a more specific inhibition, we used the kinase dead SK^{G82D} which inhibits agonist induced activation of endogenous SK [35]. The calcium peak evoked by exogenous S1P was reduced by 37±8% in cells transfected with SK^{G82D} compared to SK^{WT}, showing that activation of SK does affect calcium signaling in HEK-293 cells. Importantly, this effect is not likely mediated by S1P directly activating Ca^{2+}-release channels, since S1P was without an effect in permeabilized cells containing functional IP_3-sensitive calcium stores. Still, it cannot be ruled out that the putative S1P sensitive receptors or stores were disrupted during the permeabilisation process. However, the effect of endogenously produced S1P is most likely intracellular and not due to exported S1P acting on G-protein coupled receptors. Three observations support this assumption: 1) Supramaximal concentrations of exogenous S1P were used in this study, so exported S1P would not have an additional effect on GPCRs. 2) SK activity may be exported from the cell, but is not stimulated by agonists known to increase intracellular activity [13]. 3) In pertussis toxin treated cells uncaging of cS1P, but not stimulation with exogenous S1P leads to a calcium mobilisation.

In this study we have investigated the calcium response evoked by exogenous S1P in HEK-293 cells. In agreement with earlier work [33] we found that production of endogenous S1P indeed affects calcium mobilisation, but in contrast to these observations, we propose that the major calcium mobilizing second messenger in this system is IP_3, and that the effect of endogenously produced S1P is secondary and relatively small. Our results also suggest that the observed effect of intracellular S1P on calcium signaling in HEK-293 cells is indirect, and not through direct activation on an intracellular calcium release channel.

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