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Sphingosine-1-phosphate-activated TRPC1 channel controls chemotaxis of glioblastoma cells

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ABSTRACT

TRP channels are involved in the control of a broad range of cellular functions such as cell proliferation and motility. We investigated the gating mechanism of TRPC1 channel and its role in U251 glioblastoma cells migration in response to chemotaxis by platelet-derived growth factor (PDGF). PDGF induced an influx of Ca^{2+} that was partially inhibited after pretreatment of the cells with SKI-II, a specific inhibitor of sphingosine kinase producing sphingosine-1-P (S1P). S1P by itself also induced an entry of Ca^{2+}. Interestingly, PDGF- and S1P-induced entries of Ca^{2+} were lost in siRNA-TRPC1 treated cells. PDGF-induced chemotaxis of U251 cells was dramatically inhibited in cells treated with SKI-II. This effect was almost completely rescued by addition of synthetic S1P. Chemotaxis was also completely lost in siRNA-TRPC1 treated cells and interestingly, the rescue of migration of cells treated with SKI-II by S1P was dependent on the expression of TRPC1. Immunocytochemistry revealed that, in response to PDGF, TRPC1 translocated from inside of the cell to the front of migration (lamellipodes). This effect seemed PI3K dependent as it was inhibited by cell pre-treatment with LY294002, a PI3-kinase inhibitor.

Our results thus identify S1P as a potential activator of TRPC1, a channel involved in cell orientation during chemotaxis by PDGF.

1. Introduction

Glioblastoma multiforme (GBM), also known as grade IV astrocytoma, is the most frequent malignant primary tumour of the brain and the most aggressive cancer in humans, with a median survival of about one year [1–3]. The bad prognosis of GBM is due to its very high proliferation rate and to its invasiveness capability. Surgical debulking remains the mainstay of treatment, but the extremely infiltrative nature of the tumour makes its complete surgical removal almost impossible. Radiotherapy and chemotherapeutic agents such as temozolomide (TMZ), carmustine (BCNU) or doxorubicin (DOXO) unfortunately also present a limited efficacy. A better understanding of the molecular mechanisms that allow GBM cells to invade into the non-neoplastic brain parenchyma and to proliferate is therefore urgently needed to develop more specific and more efficient therapeutics approaches.

In GBM pathogenesis, malignant transformation and progression of the tumour to higher histological grades result from the sequential accumulation of genetic alterations of growth factor signaling pathways such as platelet-derived growth factor (PDGF) and its receptor [4,5]. The present study therefore focuses on PDGF signalling pathway and its involvement in directional migration.

It has been recently reported that Transient Receptor Potential (TRP) channels play a role in GBM cells proliferation and migration. TRP channels constitute a large family of proteins that are expressed almost ubiquitously and that mediate responses to agonists, pheromones, odorant ligands, temperature, pH, osmolarity, and oxidative stress [6]. Their activation and regulation mechanisms are very diverse and still incompletely understood [7]. Several TRP channels are involved in cell proliferation and cancer progression [8]. Other isoforms mediate apoptosis or could exert anti tumorigenic effects. A recent study reported a higher expression of TRPC1, TRPC6, TRPM2, TRPM3, TRPM7, TRPM8, TRPV1 and TRPV2 in a series of glioblastoma patients, suggesting that these TRP channels could contribute to malignant transforma-

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tion or cancer progression. At the same time, TRP overexpression was associated with increased survival in cancer patients. This suggests that TRP expression also contribute to patients' survival [9]. Among TRP isoforms, TRPM7 and TRPM8 seem to promote proliferation, invasion and/or migration of glioma cells [10]. In contrast, TRPV1 promotes cell death via endoplasmic reticulum stress pathway and is downregulated in the majority of grade IV glioblastoma [11]. TRPV2 impairs glioblastoma stem-like cell proliferation and promotes differentiation [12]. Moreover, stimulation of TRPV2 with cannabiodiol potentiates cytotoxic activity of TMZ, BCNU and DOXO [13]. Finally, it has been shown that TRPC1, the isoform studied in the present paper, enhances cell proliferation in many cancer types [14–16]. In GBM, it was reported that disruption of TRPC1 causes incomplete cytokinesis and slows cell growth [17]. TRPC1 is also involved in migration and chemotaxis [18–20]. We previously investigated the mechanisms underlying the role of TRPC1 in cell migration and showed the involvement of calcium-induced activation of calpains and proteolysis of myristoylated alanine-rich protein kinase C substrate, an actin-binding protein possibly involved in focal adhesion [19]. Besides, we showed that Ca^{2+} entry via TRPC1 also plays a role in the full activation of the PI3K/Akt pathway [16,20]. Moreover, it has been shown that TRPC1 is a crucial determinant of directionality of migration in response to chemotactic agents [21]. Indeed, stimulation of glioma cells with epidermal growth factor (EGF) results in TRPC1 channel localization to the leading edge of migrating cells and chemotaxis toward EGF was lost when TRPC1 channel was inhibited [22].

TRPC1 is a non-selective cation channel ($P_{Ca}/P_{Na} < 1$). It is involved in store-operated Ca^{2+} entry in cooperation with Orai1 where being activated by STIM1, the sensor of endoplasmic reticulum Ca^{2+} content [23–26]. However, several lines of evidence suggest that TRPC1 can be activated independently of store depletion, especially by receptor stimulation or by mechanical forces, but the gating mechanisms are still under study [27,28].

In the present study, we evaluated the gating mechanisms as well as the role of TRPC1 channel in U251 glioblastoma cells migration in response to chemoattraction by PDGF growth factor.

2. Materials and methods

2.1. Cell cultures

U251 human GBM cells were obtained from *American Type Culture Collection* (ATCC; Manassas, VA), grown in DMEM supplemented with 10% fetal bovine serum and maintained at 37 °C in a humidified atmosphere of 5% CO_2. For PDGF stimulation, cells were first serum-starved for 48 h before the beginning of the experiment and stimulated with 40 ng/ml of PDGF for the appropriate time. For $[Ca^{2+}]_i$ measurements, cells were cultured on glass coverslips.

2.2. Transfections and mRNA quantification

U251 cells were transfected with 25 nM siRNAs against human TRPC1 (siTRPC1) or human STIM1 (siSTIM1) (ON TARGET Plus smart pool, Dharmacon, Lafayette, CO, USA). Control cells were transfected by non-targeted siRNA pool (scrambled sequence; siCTRL). All transfections were performed using dharmafect-2 reagent according to manufacturer's prescriptions (Dharmacon).

Knockdown efficiency was assessed by RT-qPCR. U251 cells were homogenized in TRIzol and 2 μg of RNA was reversed-transcribed by using qScript reverse transcriptase (Quanta Biosciences). Gene-specific PCR primers were designed using Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). To avoid the amplification of genomic DNA, primers were chosen in different exons. The RNA18S and the genes of interest were amplified in parallel. Primers were purchased from Eurogentec (Seraing, Belgium). TRPC1: F, 5′- ACTGTGTAGGCATCTTCTGTGAACA-3′ and R, 5′- GGAGAAAATATACCAGAACAAAGCAAA-3′; TRPC5: F, 5′-GTCATCAAGCAAACGCT-3′ and R, 5′- AGGCTAGAGGGCATTC-3′; STIM1: F, 5′- AGCCTCAGCCATAGTCACAG –3′ and R, 5′-TTCCACATCCACATCACCATTG-3′. RNA18S: F, 5′- AGAAACG-GCTACCACATCCA –3′ and R, 5′- CACCAGACTTGCCCTCCA –3′.

Real-time RT-PCR was performed using 5 μl of cDNA, 12.5 μl of SybrGreen Mix (BioRad) and 250 μM of each primer in a total reaction volume of 25 μl. The reaction was initiated at 95 °C for 3 min, and followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 1 min and extension at 72 °C for 10 s.

Data were recorded on a DNA Engine Opticon Real-Time RT-PCR Detection System (BioRad) and cycle threshold (Ct) values for each reaction were determined using analytical software from the same manufacturer.

Each cDNA was amplified in duplicate and Ct values were averaged for each duplicate. The average Ct value for RNA18S was subtracted from the average Ct value for the gene of interest. This ΔCt value obtained in siRNA was then subtracted from the ΔCt value obtained in control conditions giving a ΔΔCt value. As amplification efficiencies of the genes of interest and RNA18S were comparable, the relation $2^{-\Delta\Delta Ct}$ gave the amount of mRNA normalized to RNA18S.

2.3. $[Ca^{2+}]_i$ measurements

Calcium measurements were performed as previously described [29,30]. Briefly, U251 cells were loaded with 1 μM Fura-2-AM (Molecular Probes) for 60 min at room temperature. Fura-2 loaded cells were excited alternatively at 340 and 380 nm and fluorescence emission was monitored at 510 nm using an AxioCam MRm camera (Zeiss) coupled to an inverted microscope (Axiovert 200 M Zeiss, objective 20X Fluar N.A. 0.75). Concentration of intracellular calcium ($[Ca^{2+}]_i$) was calculated from the ratio of the fluorescence intensities excited at the two wavelengths and after cell calibration, $\Delta[Ca^{2+}]$ measurements were calculated by subtracting the resting $[Ca^{2+}]_i$ value from the $[Ca^{2+}]_i$ peak amplitude. For each experiment (n = 1), the $[Ca^{2+}]_i$ values measured on individual cells were averaged for all cells studied on one coverslip.

2.4. Immunofluorescence assay

Cells were washed twice in PBS, fixed in 4% paraformaldehyde for 10 min and permeabilized with 0.5% triton for 5 min. Cells were then incubated in StartingBlock blocking buffer (Thermoscientific) for 1 h. TRPC1 and phospho-PDGFR^{Tyr1009} were detected by incubating the cells with anti-TRPC1 (1/75, AbCam) and anti-phospho-PDGFR^{Tyr1009} (1/50, Cell Signaling) antibodies, followed by an anti-rabbit Alexa488-conjugated secondary antibody (1/200, Invitrogen). After mounting in Prolong gold antifade reagent with 4′,6′-diamidino-2-phénylindole (DAPI, Invitrogen), the cellular localization of TRPC1 and phospho-PDGFR^{Tyr1009} was examined using a Zeiss Axiovert 100TV M microscope (20X Fluar objective, N.A. 0.75 and Apochromat 63 x objective, water immersion, NA 1.2). Images were captured using a Princeton Instruments RTE/CCD-1300-Y cooled CCD camera.

2.5. Transwell migration assay

Polycarbonate membranes (8 μm pore size) of transwell culture chambers were coated 2 h with fibronectin (5 μg/ml) and blocked 1 h by 1% inactivated BSA. 40×10^3 cells were plated on the upper surface of the membrane. Cells were allowed to migrate for 24 h.

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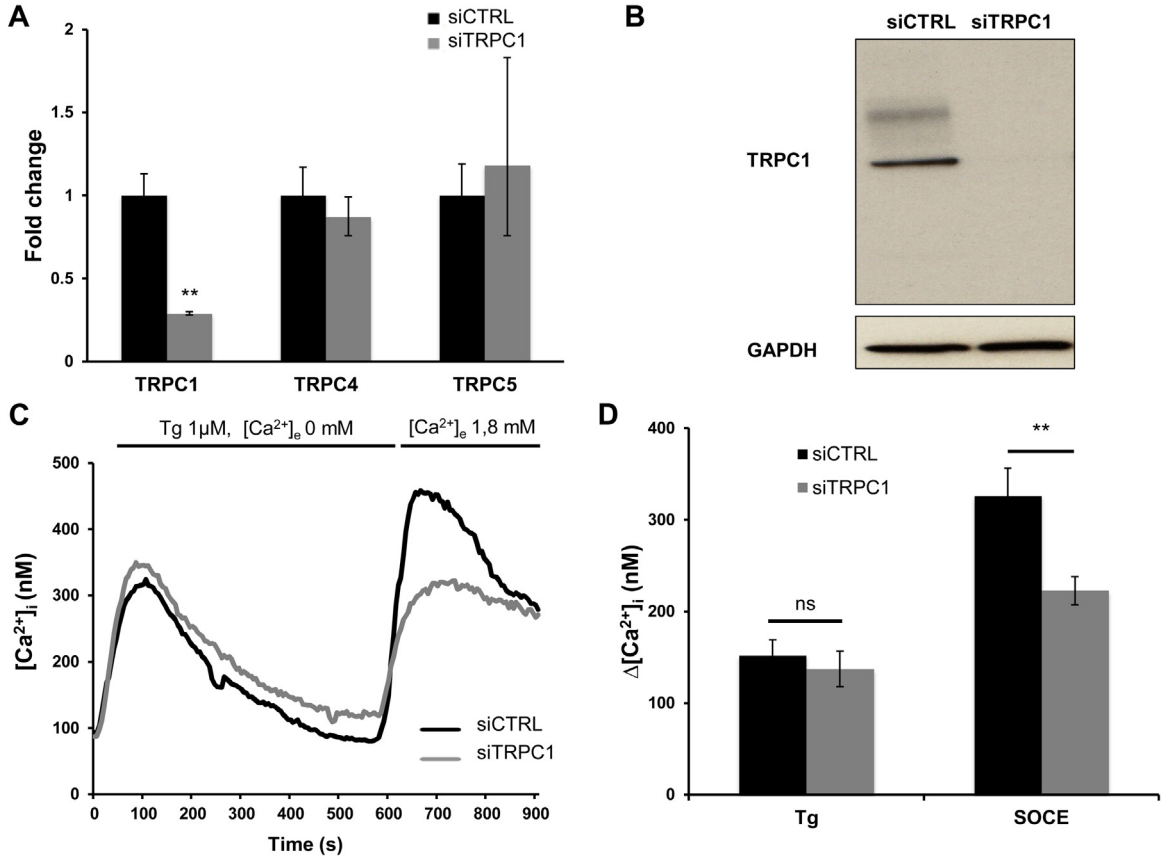

Fig. 1. Efficiency of siRNA transfection of U251 cells and the involvement of TRPC1 channel in store-operated calcium entry. A. RT-qPCR quantification of TRPC1, TRPC4 and TRPC5 mRNA expression in U251 cells after 72 h treatment with siRNA against TRPC1 (siTRPC1, grey bars) or siRNA against a scramble sequence (siCTRL, black bars) (** $p < 0.01$; $n = 7$). B. Western-blot analysis of TRPC1 protein expression after 96 h treatment with siCTRL or siTRPC1. C. $[Ca^{2+}]_i$ transients observed in response to depletion of stores with Tg (1 μM) in the absence of external Ca^{2+} (0.2 mM EGTA). Re-addition of 1.8 mM Ca^{2+} in extracellular medium induced a second peak of $[Ca^{2+}]_i$ due to SOCE in siCTRL (black curve) or siTRPC1 (grey curve) treated cells. Trace showing a representative experiment (one individual cell). D. Quantification of Tg-induced release of Ca^{2+} from ER stores and of SOCE in siCTRL (black bars) and in siTRPC1 (grey bars). Results expressed as means ± s.e.m.; Student's *t*-test: **$p < 0.01$, $n = 7$ independent experiments (7 different coverslips).

Chemoattractant PDGF was added in the lower chamber at a concentration of 40 ng/ml in serum free medium. When necessary, SKI II, LY294002 and S1P were added in the upper chamber or (when specified in the text) in both the upper and lower chambers in serum free medium. After 24 h migration, the non-migrating cells on the upper surface of the transwell were removed using a cotton swab. Then, the cells on the lower surface of the membrane were fixed in 4% paraformaldehyde, stained with Hansen's hemalun and mounted on superfrost glass slide. Six distinct fields were captured from each transwell membrane using a light microscope. The migrating cells were counted using ImageJ program. The number of migrating cells counted from 6 different fields of each membrane was averaged and considered as one independent experiment.

2.6. Cell de-adhesion

De-adhesion was measured according to the centrifugation assay described by Lotz et al. [31]. 96 wells plates were coated with fibronectin (20 μg/ml, for 2 h at room temperature), washed with PBS, and blocked with BSA (10 mg/ml). Cells suspended in serum-free medium were plated at 50×10^3 cells per well in serum-free medium containing 1 mg/ml BSA and sealed with PCR sealing film. Then, cells were seeded by centrifugation of the plates at 30*g* for 3 min at 37 °C and incubated at 37 °C for 20 min. The plates were inverted and centrifuged at 50*g* for 3 min and cells remaining attached were quantified by the acid phosphatase assay. Background of cell binding was measured in wells blocked with 10 mg/ml BSA.

2.7. In vitro wound healing assay

Cells were grown on 6 wells plates until confluence, then serum starved for 24 h before experiment. Cells were scraped off with a pipette tip to obtain a 600 μm wide acellular area (controlled in DIC microscopy). After 72 h of migration, cells were fixed and stained with Hansen's hemalun for 20 min. Image analysis and estimation of the refilling of the wound were done using the software Tscratch (CSE laboratory, ETH, Zurich, Switzerland) [32].

2.8. Western blot analysis

Cells were rinsed with PBS, scraped off in 100 μl lysis buffer containing (mM) 50 Tris/HCL (pH 7.5), 150 NaCl, 1 EDTA (pH 8), 1 EGTA, 10 ß-glycerophosphate, 1 KH_2PO_4, 1 PMSF, 1 vanadate, 50 NaF, 10 NaPPi, and 0,5% NP40, 0,5% triton, a protease inhibitor cocktail (Sigma). Cells were centrifuged at 4 °C, 10000*g* for 2 min and supernatant were kept at −80° C until use. Protein concentration was determined by the bicinchoninic acid (BCA) protein assay kit (Pierce) and absorbance was measured with a NanoDrop spectrophotometer. Samples were heated for 5 min at 95 °C in LDS sample buffer (Life technologies) containing SDS and sample reducing agent. 10 μg protein of each sample were separated on 10% SDS-polyacrylamide gels and transferred on nitrocellulose

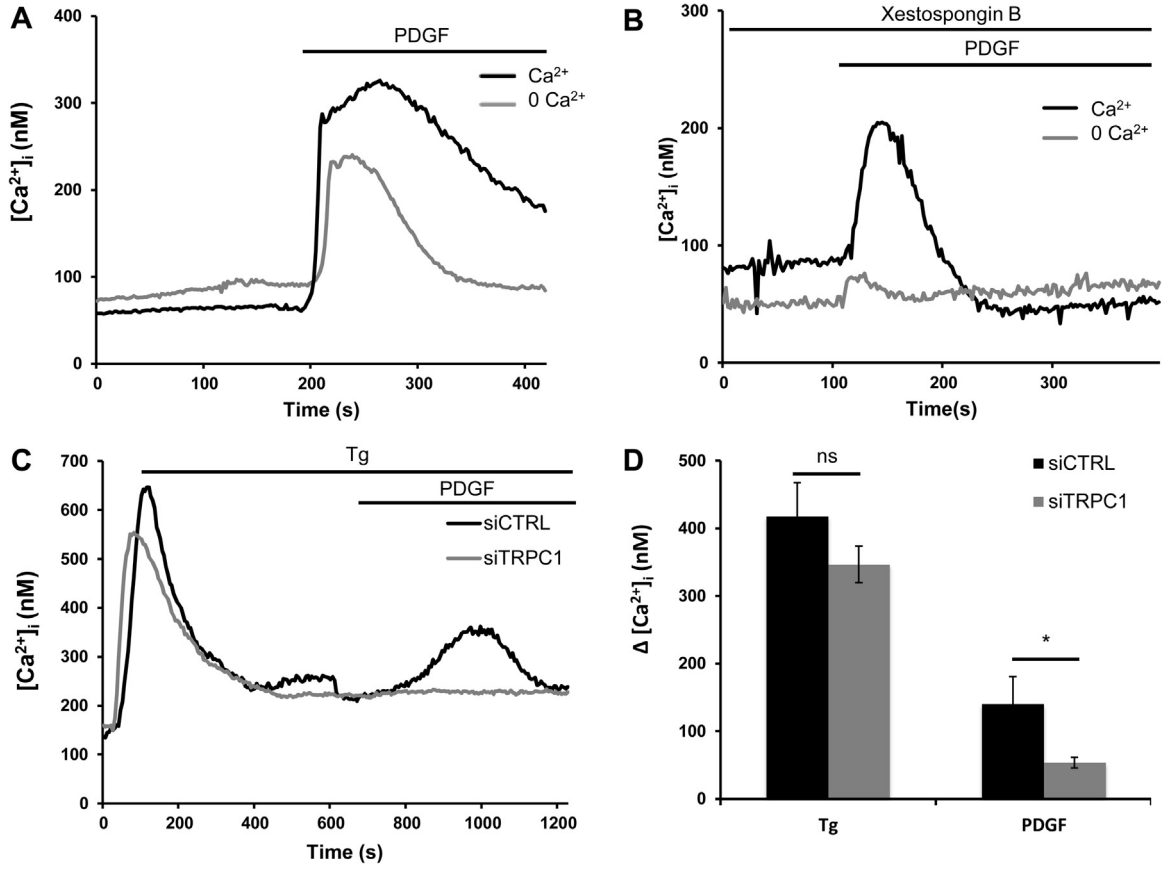

Fig. 2. Involvement of TRPC1 channel in the response to PDGF. A. $[Ca^{2+}]_i$ transient in response to 200 ng/ml PDGF stimulation in the presence (black curve) or in the absence (grey curve) of extracellular Ca^{2+}. B. Same experiment as in A, in the presence of 2 μM Xestospongin B, an inhibitor of IP3R. C. PDGF-induced store-independent Ca^{2+} entry after treatment with 1 μM Tg in the presence of extracellular Ca^{2+}. Comparison between siCTRL (black curve) and siTRPC1 (grey curve) treated cells. Trace showing a representative experiment (one individual cell). D. Quantification of Tg-releasable Ca^{2+} store and of PDGF-induced store-independent Ca^{2+} entry in siCTRL (black bars) or siTRPC1 (grey bars). Results expressed as means ± s.e.m.; Student's *t*-test: *p < 0.05 vs control, n 6.

membranes for 1 h at 100 V. After blocking with 5% non-fat milk, membranes were incubated with anti-TRPC1 (1/1000; AbCam) and anti-GAPDH (1/10000; Cell Signaling) overnight. Membranes were then incubated with secondary antibodies coupled to peroxidase (Dako) and peroxidase was detected with Pierce ECL Plus (Thermoscientific) on ECL hyperfilm. Protein expression was quantified by densitometry and reported to GAPDH expression.

2.9. Cell surface biotinylation

Treated cells were then incubated for 20 min with 1.5 mg/ml Sulfo-NHS-LC-Biotin (Pierce) in PBS (pH 8.0) on ice. Following biotin labeling, cells were washed and harvested in RIPA buffer using the same protocol as described above. Biotinylated proteins were pulled down with NeutrAvidin-linked beads (Pierce) and detected by Western blotting. Band intensities of surface proteins were obtained using Image J software.

2.10. Solutions and drugs

The Krebs solution contained (mM): 135 NaCl, 5.9 KCl, 2 $CaCl_2$, 1.2 $MgCl_2$, 10.6 Hepes Na^+ and 10 glucose (pH 7.3). In Ca^{2+} free solution, $CaCl_2$ was omitted and 0.2 mM EGTA was added. RIPA buffer was constituted of (mM): 50 Tris-HCl (pH 7.4), 150 NaCl, 2 EDTA, 1 dithiothreitol (DTT), 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 1% Triton X-100, and supplemented with Complete Protease Inhibitor Cocktail tablets (Roche Diagnostics). Thapsigargin (Tg), SKI II (inhibitor of sphingosine kinase II) and Gd^{3+} were purchased from Sigma. Fura-2/AM and FM 4-64 Dye (*N*-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium Dibromide) were purchased from Molecular probes, DMEM and serum solutions from Invitrogen and sphingosine-1-phosphate from MP Biomedicals. Pertussis Toxin, Mitomycin C and LY294002 (inhibitor of PI3K) came from Tocris Biosciences, Human PDGFbb from Cell Signaling, ruthenium red from Merck and 2-aminoethoxydiphenyl borate (2-APB) from Calbiochem. Xestospongin B was kindly given by Dr Molgo (Institut de Neurobiologie Alfred Fessard, Gif sur Yvette, France).

2.11. Statistical analysis

Data are presented as means ± s.e.m. Student's *t* tests and ANOVA were used to determine statistical significance. Statistical significance was fixed to $p < 0.05$.

3. Results

3.1. Involvement of TRPC1 channel in the response to PDGF

TRPC1 is known to form heterotetramers with TRPC3, TRPC4, TRPC5 or TRPC6 [33–35]. In U251 cells we found that TRPC1, TRPC4 and TRPC5 were by far the most expressed isoforms although a significant expression of TRPC6 has been reported by Ding and colleagues [36]. Different groups including ours have previously showed that TRPC1 is involved in store operated Ca^{2+} entry (SOCE) in many cell types [16,19]. To evaluate the involvement of TRPC1 in

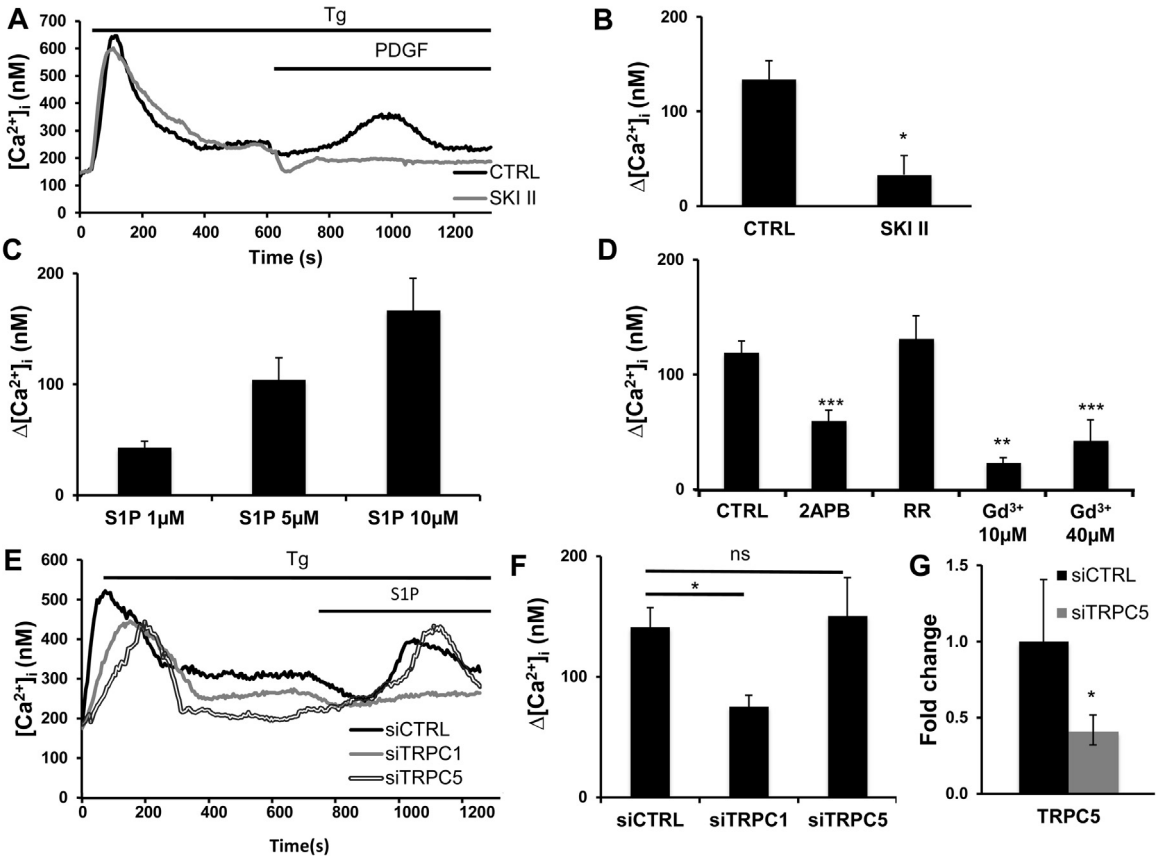

Fig. 3. S1P mediates activation of TRPC1 in response to PDGF stimulation. A. Store-independent entry of Ca^{2+} induced by PDGF in U251 cells treated (black curve) or not (gray curve) with 10 μM SKI-II during 30 min before stimulation with PDGF. B. Quantification of the PDGF-induced store-independent entry of Ca^{2+} presented in panel A. Results expressed as means ± s.e.m.; Student's *t*-test: * $p < 0.05$ vs control, n = 15 vs16. C. Dose response curve. D. Quantification of the entry of Ca^{2+} induced by 10 μM S1P in cells pretreated for 30 min with 10 μM 2APB, 10 μM or 40 μM Gd^{3+}, or with 30 μM Rutenium Red (RR). Results expressed as means ± s.e.m.; ANOVA, Newman-Keuls test: ** $p < 0.01$, *** $p < 0.001$, n = 6. E. $[Ca^{2+}]_i$ transients induced by S1P after Tg treatment in siCTRL (black curve), siTRPC1 (grey curve) and siTRPC5 (double line curve) treated cells. F. Quantification of S1P-induced store-independent entry of Ca^{2+} in siCTRL, siTRPC1 and siTRPC5 treated cells. Results expressed as means ± s.e.m.; ANOVA, Newman-Keuls test: * $p < 0.05$, n = 6 to 8. G. RT-qPCR quantification of TRPC5 mRNA expression in U251 cells after 72 h treatment with siRNA against TRPC5 (siTRPC1, grey bars) or siRNA against a scramble sequence (siCTRL, black bars) (Student's *t*-test: * $p < 0.05$ vs control, n = 11).

SOCE in U251 cells, we knocked down TRPC1 protein expression by using siRNA against TRPC1 (siTRPC1). Fig. 1A and B respectively showed a large decrease in TRPC1 mRNA and protein contents in siTRPC1 transfected cells. Note that siTRPC1 treatment did not reduce the expression level of TRPC4 and TRPC5. In the absence of extracellular Ca^{2+}, treatment of the cells with 1 μM thapsigargin (Tg) induced a release of Ca^{2+} from the ER stores to a similar extent in siCTRL and siTRPC1 cells (Fig. 1C). Re-addition of 1.8 mM Ca^{2+} into the extracellular medium induced a large entry of Ca^{2+} in siCTRL cells that was slightly but significantly smaller in siTRPC1 cells (Fig. 1C quantified in 1D). These results indicate a modest implication of TRPC1 channel in SOCE in U251 cells.

Binding of PDGF to its receptor activates PLC-γ that produces IP3 and triggers the release of Ca^{2+} from the ER. As expected, treatment of U251 cells with 200 ng/ml PDGF induced a Ca^{2+} transient that declined faster in the absence of extracellular Ca^{2+} than in its presence, an effect presumably due to a SOCE (Fig. 2A). However, when the release of Ca^{2+} was completely inhibited by a pre-treatment of the cells with 2 μM Xestospongin B, a highly specific inhibitor of IP3R [37], PDGF was still able to induce an entry of Ca^{2+} (Fig. 2B), suggesting that PDGF also induces a store-independent entry of Ca^{2+}. Moreover, when cells were first treated with Tg in the presence of extracellular Ca^{2+} and PDGF was added after the SOCE, i.e. when $[Ca^{2+}]_i$ had returned to an intermediate value around 200 nM, PDGF still induced a Ca^{2+} response (Fig. 2C quantified in 2D). This suggests that PDGF is able to induce a store-independent entry of Ca^{2+}. Importantly, the influx of Ca^{2+} was dramatically reduced in TRPC1-depleted cells.

3.2. S1P mediates store-independent activation of TRPC1 in response to PDGF stimulation

The results described above prompted us to investigate the mechanism underlying TRPC1 activation upon PDGF stimulation. Several signal transduction pathways are triggered in response to PDGFR activation. Among the second messengers produced, sphingosine-1-phosphate (S1P), a biolipid produced by sphingosine phosphorylation by sphingosine kinase mediates mitogenic effects of PDGF [38]. We therefore investigated whether S1P may participate to the store-independent activation of TRPC1 upon PDGF stimulation. Using the protocol described above in Fig. 2C (Tg treatment in the presence of extracellular Ca^{2+}), we observed that treatment of U251 cells with 10 μM SKI II, a common used inhibitor of sphingosine kinase almost abolished the store-independent entry of Ca^{2+} induced by PDGF (Fig. 3A,B). In contrast, SKI II did not modify Tg-induced $[Ca^{2+}]_i$ increase. In agreement with these observations, PDGF response could be mimicked by direct stimulation of the cell with 1 to 10 μM S1P (Fig. 3C). S1P-induced Ca^{2+} entry response was inhibited by different non-specific inhibitors of TRPC channels such as 10 μM 2-APB (a membrane permeant inhibitor of IP3R and TRPC channels but an activator of some TRPV isoforms [39]) or 10 μM or 40 μM Gd^{3+} but not after treatment

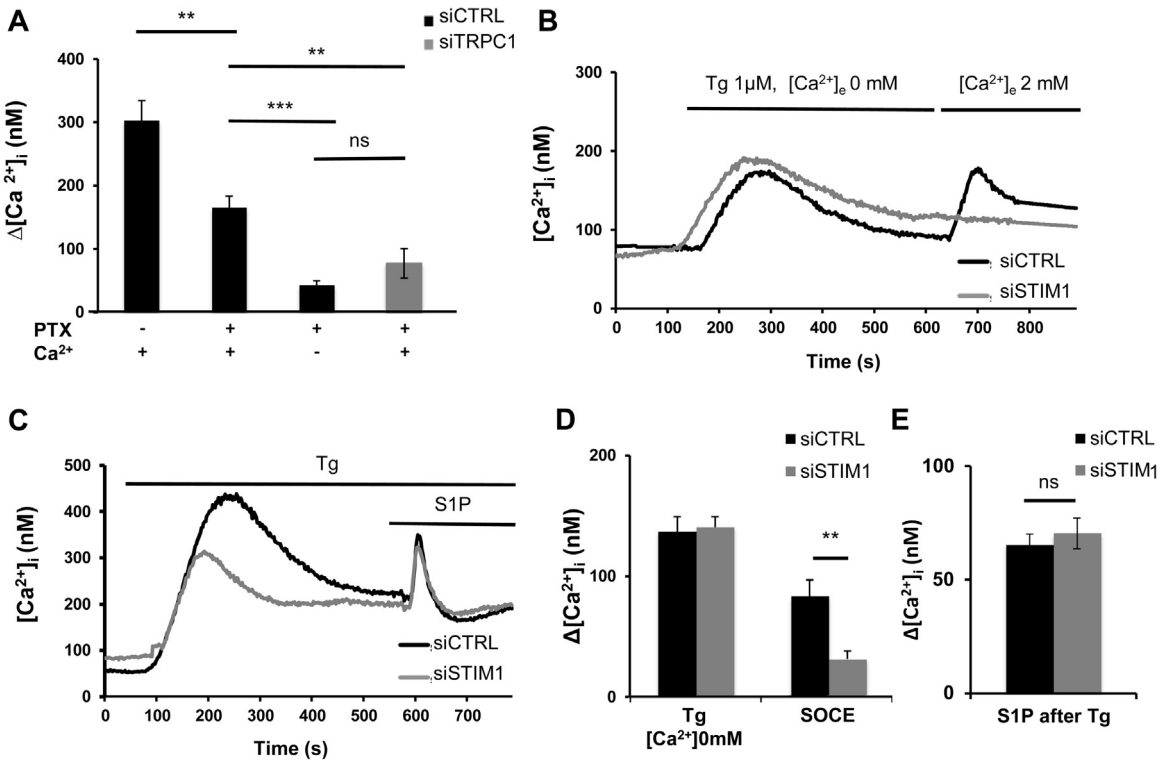

Fig. 4. S1P-mediated activation of TRPC1 is store-independent. A. Quantification of Ca^{2+} entry after stimulation by S1P-induced entry of Ca^{2+} in siCTRL (black bars) or siTRPC1 (grey bars) cells treated with 100 ng/ml PTX, an inhibitor of Gi/o protein coupled receptors in the presence or in the absence of external Ca^{2+}. Results expressed as means ± SEM; ANOVA, Newman-keuls test **p < 0.01, ***p < 0.001, n indicated in the figure. B. $[Ca^{2+}]_i$ transients observed in response to depletion of stores with Tg (1 μM) in the absence of external Ca^{2+} (0.2 mM EGTA). Re-addition of 1.8 mM Ca^{2+} in extracellular medium induced a second peak of $[Ca^{2+}]_i$ due to SOCE in siCTRL (black curve) or siSTIM1 (grey curve) treated cells. C. S1P-induced entry of Ca^{2+} after Tg treatment in the presence of extracellular Ca^{2+} in siSTIM1 treated cells. D. Quantification of experiments presented in panel B. E. Quantification of experiments presented in panel C. Results expressed as means ± s.e.m.; Student's *t*-test: **p < 0.01, n = 5.

with ruthenium red (a well known inhibitor of TRPV channels [40] but also of ryanodine receptors [41]) (Fig. 3D). Specific inhibition of TRPC1 expression by siTRPC1 treatment significantly decreased the response to S1P (Fig. 3E,F) without modifying the Tg-induced release of Ca^{2+}. These results suggest that PDGF-induced store-independent entry of Ca^{2+} is mediated by the action of S1P on TRPC1. However, it has been reported that S1P activates TRPC5 ion channel, an isoform that is able to form heterotetramers with TRPC1. To investigate whether the response to S1P involved TRPC5, we knocked down TRPC5 in U251 cells and measured Ca^{2+} response to S1P after Tg pre-treatment. In spite of an efficient repression of TRPC5, the response to S1P was unaffected, indicating that TRPC5 is not essential in the response to S1P (Fig. 3F,G).

As a lipid, S1P produced in the cell can diffuse out of the cell and exert autocrine or paracrine effects by acting on its membrane receptors. These receptors are coupled to G-proteins, some of which activate PLC, Ca^{2+} release from the stores and subsequent SOCE. We then investigated the eventual involvement of S1P receptors in the store-independent entry of Ca^{2+} in response to S1P. We first treated U251 cells with 100 ng/ml pertussis toxin (PTX), a drug that inhibits Gi/o family of G proteins, and observed a complete abolition of Ca^{2+} release from the stores (Fig. 4A, third bar of the histogram). However, in the presence of PTX, S1P was still able to induce an entry of Ca^{2+} that was significantly inhibited in siTRPC1 treated cells (Fig. 4A, compare second and fourth bars of the histogram). These results again suggest that S1P is able to trigger an entry of Ca^{2+} independently of store depletion, and independently to Gi/o coupled S1P receptors (S1PR).

As described above, TRPC1 is a component of SOCE in many cell types as well as in U251 glioblastoma cells. To definitively exclude the involvement of SOCE in the S1P-dependent activation of TRPC1 in U251 cells, we knocked down STIM1 and measured the response to S1P. We first confirmed that repression of STIM1 protein expression largely reduced SOCE (Fig. 4B–D). Indeed, $[Ca^{2+}]_i$ transient observed upon re-addition of external Ca^{2+} on cells pre-treated with Tg was abolished (Fig. 4B). Similarly, $[Ca^{2+}]_i$ transient observed when Tg was applied in the presence of Ca^{2+} was also reduced (Fig. 4C). However, the amplitude of the store-independent entry of Ca^{2+} induced by S1P was not reduced in siSTIM1 treated cells (Fig. 4C,D).

3.3. *S1P activates TRPC1 during directional migration independently of S1PR*

We next investigated the functional role of the specific activation of TRPC1 by S1P in U251 cells migration. Directional migration was studied by measuring the chemotactic response of siCTRL and siTRPC1 cells to PDGF using modified Boyden chambers. As shown in Fig. 5A and quantified in Fig. 5B, directional migration was dramatically reduced in siTRPC1 cells. Similar results were obtained with a second siRNA against TRPC1, excluding off target effects (data not shown). To eliminate a possible effect of TRPC1 on proliferation, we repeated the experiments in the presence of 30 μM mitomycin C, an inhibitor of DNA synthesis, and obtained a similar reduction of chemotactic response of siTRPC1 cells to PDGF (Fig. 5A quantified in 5C).

To investigate the sphingosine kinase pathway in the chemoattraction by PDGF, we treated U251 cells with 10 μM SKI II and compared the rate of directional migration. We observed that SKI II inhibited the number of migrating cells by a factor 3 (Fig. 5D). To exclude a non-specific toxic effect of SKI II, we tried to rescue directional migration by adding 10 μM S1P to cells treated with SKI

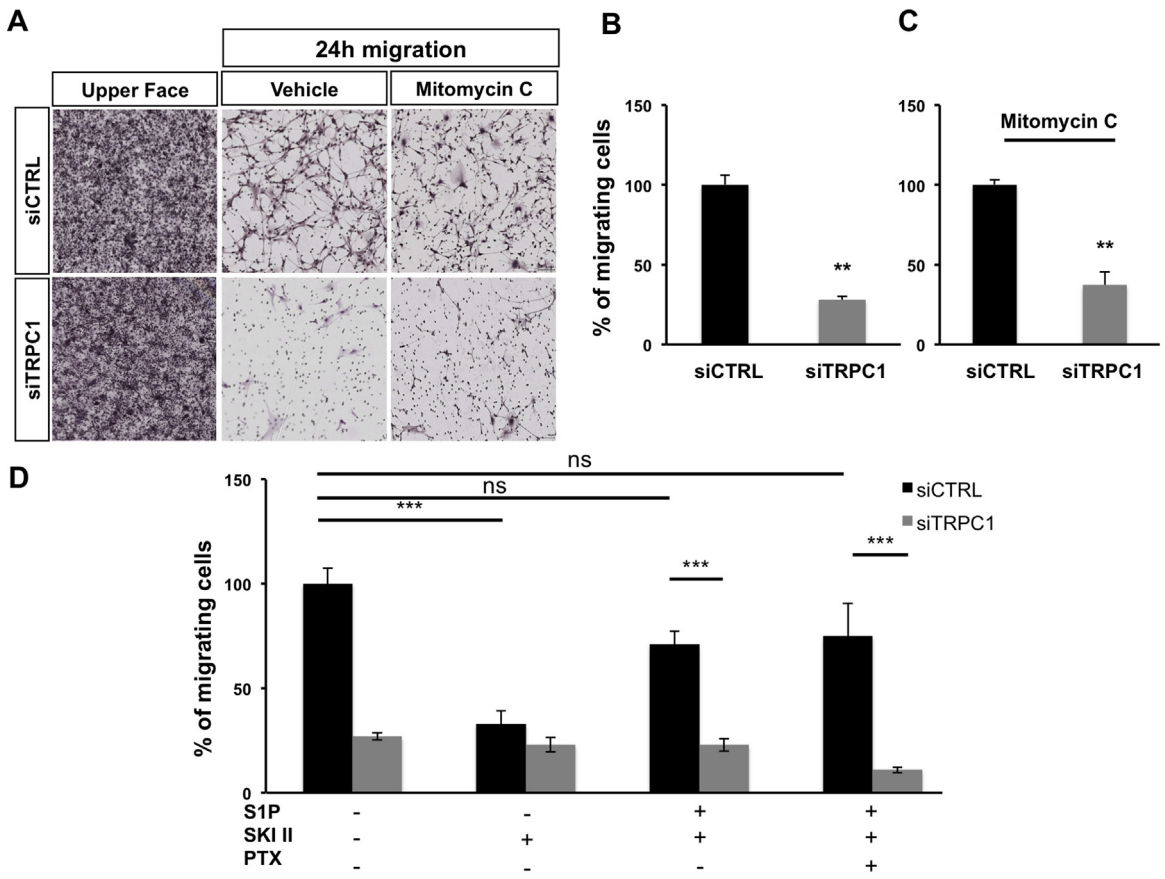

Fig. 5. S1P activates TRPC1 during directional migration independently of S1PR. A. Cell migration analysis of U251 cells treated with siTRPC1 or siCTRL as gauged by Boyden chamber assay. The pictures on the left panels show U251 cells (confluent culture) on the upper face and on the middle and right panels, cells having migrated from the upper face to the lower face 24 h after PDGF (40 ng/ml) stimulation (lower compartment). B and C. Quantification of migrating cells after 24 h of chemoattraction by 40 ng/ml PDGF with (B) or without 40 μg/ml mitomycin C (C). Results expressed as means ± s.e.m. Student *t*-test **p < 0,01, n = 5. D. Percentage of migrating cells after 24 h treatment with 40 ng/ml PDGF, 10 μM SKI-II, 1 μM S1P or 100 ng/ml PTX in siCTRL (black bars) or siTRPC1 (grey bars) treated cells. Results expressed as means ± s.e.m.; ANOVA, Newman-Keuls test: ***p < 0.001, n = 5.

II. Similar results were obtained whether SKI II and S1P were added in the upper chamber only or whether they were added to both the upper and the lower chambers, suggesting that S1P does not exert a repellent effect. Interestingly, S1P treatment rescued directional migration of SKI II treated cells only when TRPC1 was expressed. Finally, PTX treatment did not change the rescue of directional migration by S1P, suggesting that the action of S1P on migration did not depend on Gi/o protein-coupled S1P receptors.

Taken together, these results suggest that chemotactic response of U251 glioblastoma cells to PDGF is mediated by the action of S1P on TRPC1.

3.4. TRPC1 does not play a major role in 2D planar migration of U251 glioblastoma cells

We previously reported the involvement of TRPC1 in 2D planar cell migration in myoblasts, an effect attributed to a change in adhesion process [19]. Here, we investigated 2D migration of glioma cells by using a wound-healing assay. In the absence of growth factors, we observed a basal level of migration that was similar in siCTRL and siTRPC1 treated cells. When stimulated with 40 ng/ml PDGF, there was a slight but significant decrease of migration in siTRPC1 cells compared to controls (Fig. 6A quantified in 6B). We then studied adhesion process. As shown Fig. 6C, adhesion of U251 cells to fibronectin-coated coverslips was complete after 50 min. We therefore compared adhesion of siCTRL vs siTRPC1 treated cells after 20 min and observed a very modest increase of adhesion in siTRPC1 treated cells (Fig. 6D).

3.5. TRPC1 translocates to the lamellipodia during U251 chemotaxis by PDGF

We showed that TRPC1 activation by S1P is indispensable for U251 cells chemotaxis in response to PDGF. In serum free medium, immunodetection of TRPC1 revealed a diffuse signal localized throughout the cell (Fig. 7A). To verify the specificity of the antibody, we checked that no signal or less was found in siTRPC1-transfected cells (Fig. 7B). In response to PDGF stimulation, TRPC1 was clearly localized in the front of migration (Fig. 7A,C,D). As a control, PDGFR was localized in the cellular membrane in serum free conditions and did not present any major change when the cells were stimulated with PDGF. When the plasma membrane was stained with the lipophilic dye FM 4-64, the whole membrane was stained and the dye did not only accumulate in the leading edge of lamillipodium, suggesting that the apparent concentration of TRPC1 does not artifactually result from membrane ruffling activity in this zone but reflects a specific targeting of the channel to the leading edge of lamellipodia during migration (Fig. 7C). As PI3K, a downstream signalling pathway of PDGFR, is a well-known regulator of membrane protein translocation, we treated U251 cells with 25 μM LY294002, an inhibitor of PI3K, and checked for TRPC1 localisation. As shown in Fig. 7, the targeting of TRPC1 to cell lamellipodia induced by PDGF was dramatically reduced in cells

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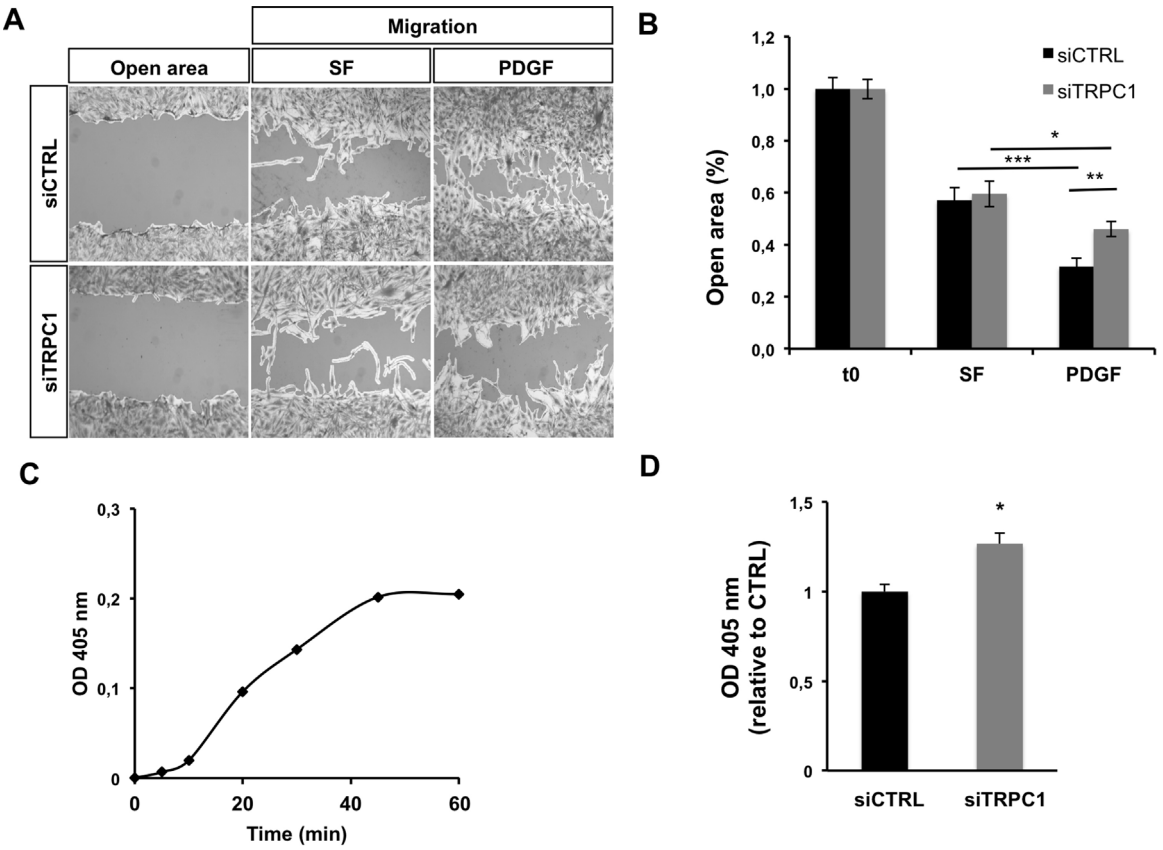

Fig. 6. TRPC1 does not play a major role in 2D planar migration of U251 glioblastoma cells. A. Representative images of cell migration before and after wound healing. Cells were transfected with siCTRL or siTRPC1, were serum starved 24 h before the test and were stimulated or not with 40 ng/ml PDGF during 72 h. B. Quantification of wound area expressed as a percentage of total image area after 24 h of cell migration. Results expressed as means ± s.e.m.; ANOVA, Newman-Keuls test: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, n = 8. C. Time course of untransfected U251 cell adhesion. D. Quantification of adhesion after 20 min for siCTRL or siTRPC1 treated U251 cells. Results expressed as means ± s.e.m., Student's *t*-test: $^{*}p < 0.05$, n = 12.

treated with LY294002. PDGFR immunodetection stayed however unchanged in the presence of LY294002 as compared to control. As a consequence, treatment of the cells with 25 μM LY294002 almost completely inhibited PDGF-induced chemotaxis (median residual migration of 15% of the control, n = 3). However, it is important to note that LY294002 also inhibited 2D-migration but to a lesser extent (by 30 to 40%).

Finally we used a biotinylation technique to examine the effect of PDGF on cell surface expression of TRPC1. The biotinylated fraction of TRPC1 was low at rest, but significantly increased by a factor of 4.6 after PDGF stimulation (Fig. 8), suggesting an insertion of TRPC1 in the plasma membrane.

4. Discussion

The gating mechanisms of TRP channels seem to be extremely diverse [27]. This is particularly true for TRPC1 which is reputed to be activated by Ca^{2+} stores-depletion [16,23,24] but which is also involved in receptor-activated Ca^{2+} entry pathway requiring IP_3 receptor activation [42]. TRPC1 can also be directly activated by PIP_3, by phosphorylation by PKC [43,44] or through the hydrolysis of PIP_2 and/or concomitant generation of DAG [45] or other lipids (see below). Finally, TRPC1 has also been reported to be mechanosensitive [46], although this has been disputed [47–49]. Such a diversity of modulating and gating mechanisms probably reflects the molecular heterogeneity of ion channels involving TRPC1. Indeed, TRPC1 forms functional heteromultimers with TRPC3 in transfected or native HEK293 cells or in the embryonic brain and with TRPC4 and/or TRPC5 in transfected cells or in adult brain [50,51]. It also interacts with TRPP2, TRPV4 and TRPV6 channels, and with Orai1 and STIM1 that constitute the CRAC channel. The polymodality of TRPC1 might also result from its interaction with membrane receptors such as metabotropic glutamate receptor 1 or fibroblast growth factor receptor, and with cytosolic factors such as calmodulin, caveolin and homer proteins (reviewed in [27]).

All the experiments conducted in the present study converge to show that PDGF induces a store-independent entry of Ca^{2+} in U251 glioblastoma cells, which is mediated by the action of S1P on TRPC1.

Different lipids have been shown to stimulate or modulate TRPC channel function. Besides DAG already mentioned that stimulates TRPC3/TRPC6/TRPC7 [52], lysophospholipids such as lysophosphatidylcholine generated by the action of phospholipase A2 (PLA2) on phosphatidylcholine have been shown to stimulate TRPC5 and TRPC6. Arachidonic acid and its metabolites activate TRPC4, TRPC6 and TRPC7 whereas cholesterol and derivatives exert their effects on TRPC3 or indirectly on TRPC1 by promoting its localisation on lipid rafts (see [53] for review). Lipid second messengers might be produced by membrane stretching and could therefore mediate mechanosensitivity to different TRP isoforms. For example, mechanical stimuli are sensed by PLA2, leading to the production of arachidonic acid which is in turn metabolized by P450 epoxyoxidases to epoxyeicosatrienoic acids (EETs) that are able to stimulate TRPV4 [54]. In skeletal muscle cells, S1P has also been reported to modulate stretch-induced Ca^{2+} influx through a channel constituted, at least partially of TRPC1, although the mechanosensitivity of TRPC1 has been contested [46,48,55].

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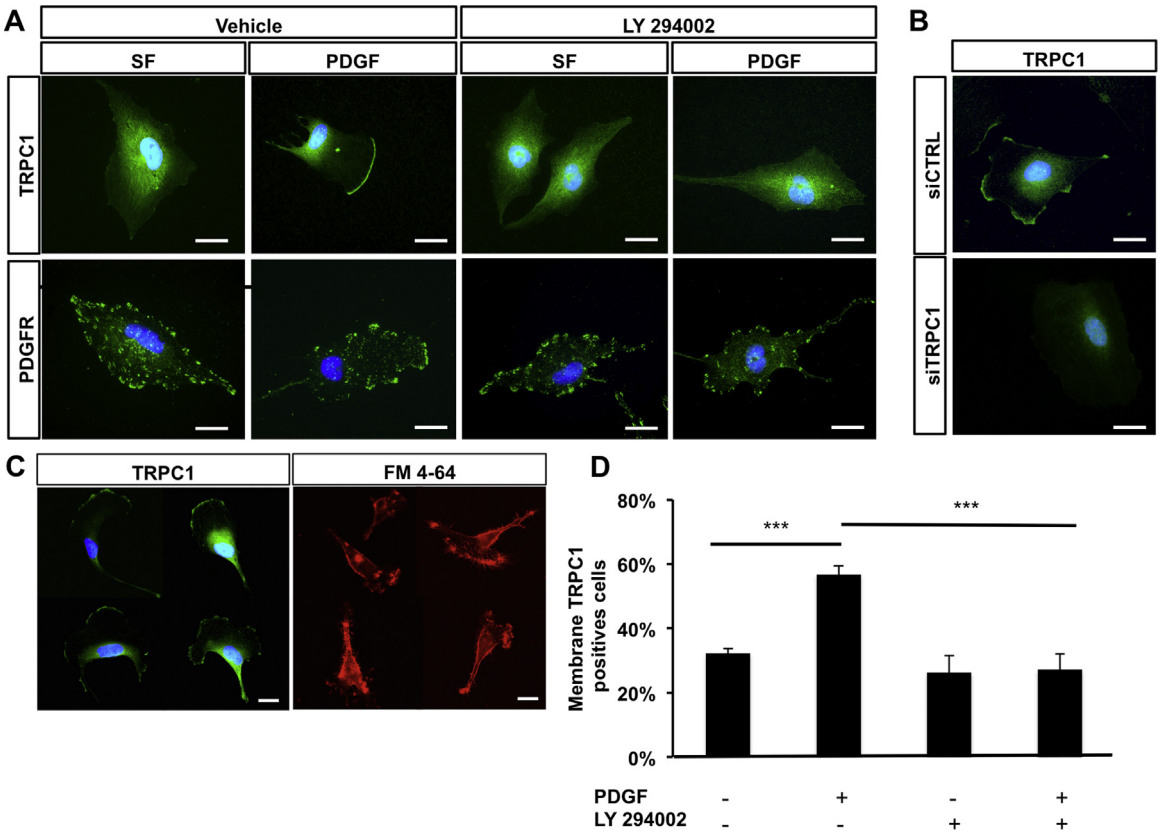

Fig. 7. TRPC1 translocates to the lamellipodia during U251 chemoattraction by PDGF. A and B. Immunocytochemistry of U251 after fixation and staining with anti-TRPC1 (upper panel A and B) and with anti-PDGFR (lower panel A) antibodies and with Hoechst. Scale bars represent 10 μm. In panel A, cells were serum starved for 24 h before experiments. Before fixation, U251 were stimulated 48 h with 40 ng/ml PDGF in the presence or in the absence of 25 μM LY294002. B. Comparison of TRPC1 expression and localization in siCTRL- and siTRPC1-treated cells. C. Cells cultured in the presence of PDGF labeled with FM 4-64 lipophilic dye or immune-stained with anti-TRPC1 antibody. D. Quantification of membrane TRPC1 positive cells when they are treated with 40 ng/ml PDGF or/and LY294002 (25 μM) (counting in blind). Results expressed as means ± s.e.m.; ANOVA, Newman-Keuls test: ***$p < 0.001$, $n = 5$.

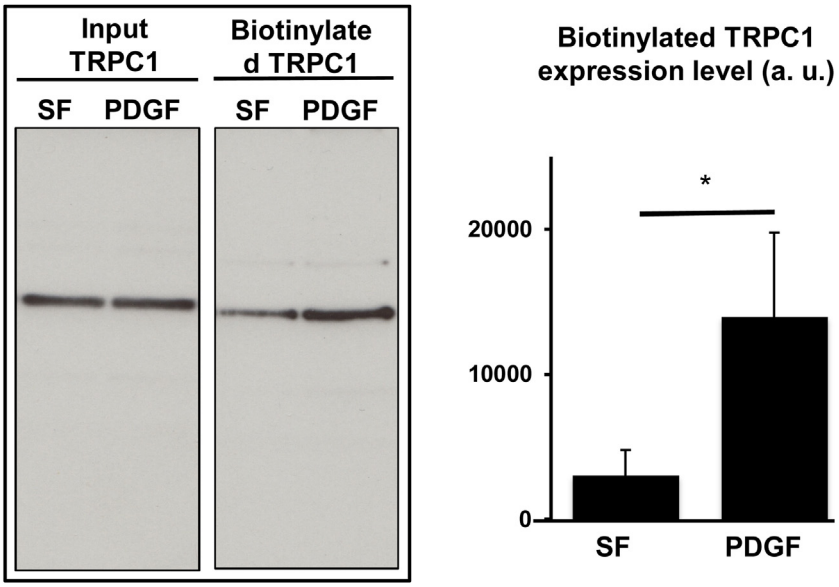

Fig. 8. Translocation of TRPC1 to the plasma membrane. Detection of biotinylated TRPC1 in U251 cells kept in serum free (SF) condition (first lane) or treated for 24 h with 40 ng/ml PDGF (second lane), before (upper western blot) or after pull down (lower western blot) with NeutrAvidin-linked beads. Lower panel: quantification of biotinylated TRPC1 by densitometry (arbitrary units). The individual results are plotted and the median is indicated; Mann-Whitney test: *$p < 0.05$, $n = 4$.

The group of David Beech clearly showed that in smooth muscle cells, S1P activated TRPC5 ion channel via two mechanisms, one intracellular and one extracellular, involving G-protein-coupled receptor and completely inhibited by pertussis toxin [56]. As TRPC5 is able to form heterotetramers with TRPC1, we used different strategies to investigate whether S1P exerted its action on TRPC1

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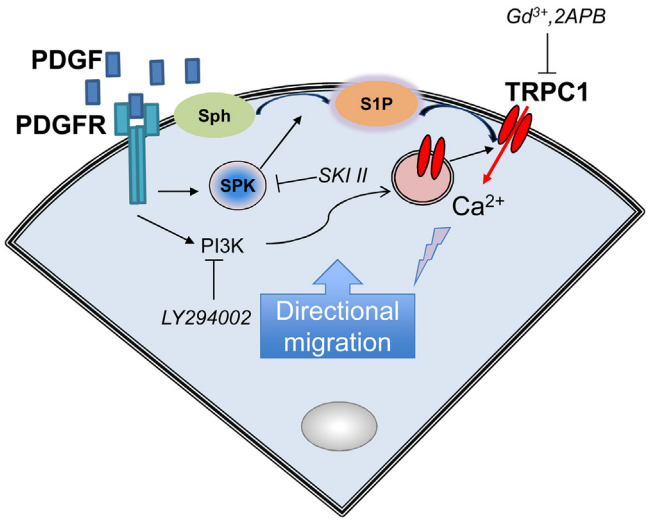

Fig. 9. Schematic actions of PDGF and S1P on TRPC1-mediated entry of Ca^{2+}. PI3 K: PI3 kinase; PDGF: Platelet-derived growth factor; PDGFR: PDGF receptor; S1P: sphingosine-1-phosphate; SKI II: sphingosine kinase inhibitor II; SPK: sphingosine kinase; Sph: sphingosine.

or on TRPC5 or both. First, we treated control U251 cells with 10 μM Gd^{3+}, a concentration at which TRPC5 is normally activated whereas TRPC1 is inhibited. As shown in Fig. 3D, at this concentration of Gd^{3+}, the store-independent Ca^{2+} response to S1P was inhibited. Second, we checked for a possible off target effect of siTRPC1 RNA strategy. As seen in Fig. 1A, the level of TRPC5 was not reduced in siTRPC1-treated cells, suggesting that the loss of effect of S1P was not due to indirect decrease of TRPC5. Third, we knocked down TRPC5 in U251 cells and checked for the Ca^{2+} response to S1P after Tg pre-treatment. TRPC5 was indeed well repressed in siTRPC5-treated U251 cells but the store-independent Ca^{2+} response to S1P was similar to that observed in siCTRL cells (Fig. 3E and F). Beside, in contrast to the situation observed in smooth muscle [56], when added extracellularly, S1P activated a TRPC1-dependent entry of Ca^{2+} that was not completely inhibited by pertussis toxin. All these experiments reinforce our conclusion that in U251 glioblastoma cells, S1P triggers a TRPC1-dependent and store-independent entry of Ca^{2+}. Whether S1P directly or indirectly interferes with TRPC1 channel remains to be determined.

TRPC1 channel has been involved in the migration of several cell types. This is due to a dual effect on adhesion and on chemotaxis [18,19,21,57,58]. Here we show that its role in PDGF-induced chemotaxis is dependent on both its targeting to the leading edge of lamellipodia and its activation by S1P. Indeed, both the inhibition of the PI3K by LY294002 and inhibition of PDGF-induced S1P production by SKI-II inhibited chemotaxis. The latter effect was rescued by addition of S1P. PI3K inhibition by LY294002 also inhibited 2D-migration. This probably reflects the fact that PI3K/Akt module acts at the leading edge of the cell and regulates the accumulation of phosphatidylinositol 3,4,5-trisphosphate (PIP3) and in turn cytoskeletal activities involved in cell protrusion [59]. Note also that LY294002 not only binds to class I PI3Ks and other PI3K-related kinases, but also to proteins unrelated to the PI3K family such as mTOR or GSK3 [60].

Hofmann et al. nicely demonstrated that TRPC1 homotetrameric channels do not translocate to the plasma membrane [61]. TRPC1 however co-assembles with all members of the TRPC subfamily and forms heterotetramers with a lower Ca^{2+} permeability than homotetramers [34]. In GnRH neurons, TRPC1 repression induces an increase of Ca^{2+} permeability of heterotetramers pre-existing in the membrane and has a positive effect on migration [34]. This effect is in apparent contradiction with the results presented here. This might be due to the absence of TRPC1 in the membrane in resting conditions in U251 cells (Fig. 7) and to the PDGF-induced insertion in the membrane of new heterotetramers allowing Na^+ and Ca^{2+} influxes (Fig. 7, 8). However, we do not know here whether TRPC1 targeted to the membrane forms or not heterotetramers in U251 cells. Besides, a longer version of TRPC1 with 78 additional amino acids at the N-terminus was recently identified by Ong and colleagues [62]. This isoform might behave differently than shorter isoforms studied so far.

In conclusion, our results suggest that PDGF induces the formation of lamellipodia and promotes PI3K-mediated targeting of TRPC1 to the leading edge, and at the same time activates sphingosine kinase that forms S1P, which in turn activates TRPC1 (Fig. 9). Entry of Ca^{2+} through TRPC1-containing channels might then induce a $[Ca^{2+}]_i$ gradient in the cell, allowing it to move in a specific direction [21].

The capacity of glioblastoma to diffusely invade the normal brain parenchyma makes these tumours difficult to resect and is therefore partially responsible of their poor prognosis. PDGF and EGF receptors are overexpressed in a high percentage of glioblastoma and represent therapeutic targets to combat these tumours [63]. Specific pharmacological inhibitors of TRPC1 or of sphingosine kinase or PI3 K might represent interesting alternatives to stem glioblastoma invasion. These observations should encourage further studies of these signalling pathways.

Conflict of interest

The authors declare no competing financial interests.

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