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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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