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Initiation of Oligodendrocyte Progenitor Cell Migration by a PDGF-A Activated Extracellular Regulated Kinase (ERK) Signaling Pathway

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Abstract

During CNS development, oligodendrocyte progenitor (OP) cells migrate from germinal zones to presumptive white matter tracts to generate myelinating oligodendrocytes. In vitro and in vivo studies indicate that platelet-derived growth factor-A (PDGF-A) is a potent chemoattractant for OP cells and important for normal distribution throughout the developing CNS. However, PDGF-A does not localize in concentration gradients corresponding to OP migratory pathways, as would be expected for a chemoattractant to direct migration. Therefore, the mechanism by which PDGF-A regulates OP distribution remains to be clarified. Here we show that PDGF-A induces OP migration and continuous exposure to PDGF-A is not required to maintain migration. Using pharmacological inhibitors, we show that a self-sustaining extracellular-regulated-kinase signaling pathway drives OP migration for up to 72 hours after the initial PDGF stimulus. These findings indicate PDGF-A may act to mobilize OP cells that then respond to distinct directional signals to distribute appropriately within the CNS.

Keywords

Oligodendrocyte progenitors; PDGF receptor; ERK signaling; Migration

Introduction

Oligodendrocytes (OLs), the myelinating cells of the CNS, originate as platelet-derived growth factor alpha-receptor (PDGFRα) expressing oligodendrocyte progenitor (OP) cells, in restricted areas throughout the developing CNS. Their initial appearance in these discrete ventricular areas is regulated by local signals such as sonic hedgehog [1]. During the

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development of the CNS, OP cells then migrate significant distances to populate presumptive white matter [2]. The complex regulatory mechanisms underlying the migration, proliferation and differentiation of OP cells remain to be clearly defined. Several factors are known to play a significant role in OP distribution during brain development, including Netrin-1, which acts to drive OP cells away from the germinal matrix [3]; CXCL1, which acts to stop OP migration and initiate proliferation [4]; extracellular matrix proteins [5] and axonal processes [6].

PDGF-A is a potent mitogen for OP cells and is known to play a crucial role in normal embryogenesis [7,8]. PDGF-$A^{-/-}$ mouse embryos have significantly reduced numbers of PDGFRα-positive OP cells [7] and those embryos that survive postnatally are severely hypomyelinated [9]. Interestingly, the loss of OP cells and myelin is most marked at the periphery of the brain and spinal cord, i.e. at sites most distant from the presumed sites of origin of OP cells [10] suggestive of a migratory defect. Further, the optic nerves of PDGF-$A^{-/-}$ mice had few myelinated fibers close to the chiasm and a complete lack of myelin at the retinal end [10]. These findings provide evidence that PDGF-A plays an important role in the distribution of OP cells during CNS development.

PDGF-A has been shown to act as an OP chemoattractant in vitro. However, there is little evidence to support the presence of a PDGF-A chemotactic-gradient in the developing CNS. Recent studies of the role of Netrin-1 as a chemorepellent to drive the OP cells away from the germinal matrix provide compelling evidence that chemorepulsion may direct migration rather than a chemoattractant gradient [3,11]. Therefore, how PDGF signaling impacts OP migration is not yet clear. Activation of the PDGFRα (a tyrosine kinase receptor) results in the activation of numerous downstream signaling pathways including phosphoinositide-3 kinase (PI_3 K), mitogen activated kinases (MAPK) and phospholipase C gamma (PLCγ). Previous studies have shown a critical role for the PI_3 K pathway in regulating OP proliferation [12], as well as a role for p^{38} MAPK in OL differentiation [13]. OP migration is a prerequisite for myelination, and yet there have been few studies of signaling pathways responsible for migration in OP.

We examined the hypothesis that PDGF-A regulates OP migration via the extracellular regulated kinase (ERK) signaling pathway. We show that transient exposure (30 min) of OP cells to PDGF-A stimulates OP migration via the ERK signaling pathway. Further, a 30-min pulse of PDGF-A is sufficient to stimulate continuous OP migration for a period of at least 72 h without inducing proliferation. In addition, we show that short-term exposure to PDGF-A results in the activation of a self-regulating positive feedback loop of the ERK signaling pathway [14] which is involved in the sustained migration of OP cells. This signaling mechanism may allow PDGF to initiate OP migration, which is then directed by a complex interaction of chemoattractive and chemorepulsive signals.

Materials and Methods

Materials

All materials were from Sigma (St. Louis, MO, USA) unless otherwise stated. Platelet derived growth factor-AA (PDGF-A) was from R&D Systems (Minneapolis, MN, USA). Bromodeoxyuridine (BrdU) was from Boehringer Mannheim (Roche, Palo Alto, CA, USA). Monoclonal anti-BrdU antibody was from Beckton Dickinson, (San Jose, CA, USA). Polyclonal NG2 antibody was a generous gift of Dr. Joel Levine. Monoclonal phospho-ERK antibody (for ICC) was from Cell Signaling. Secondary antibodies used were from Jackson ImmunoResearch (West Grove, PA, USA), donkey anti-mouse IgG (H + L) F(ab')2 conjugated with FITC and donkey anti-rabbit IgG conjugated with FITC. The pharmacological inhibitors used were PD098059 from Cell Signaling Technology (Beverly, MA, USA); LY294002, SB203580, U0126, and $AACOCF_3$ were from CalBiochem (San Diego, CA, USA). Antibodies used for Western blot were: rabbit polyclonal anti-phospho-ERK1/ERK2 (T202/Y204) affinity

purified (R&D Systems, Minneapolis, MN), rabbit polyclonal anti-mitogen activated protein kinase (ERK-1/2) (Sigma), rabbit polyclonal anti-ERK2 (AbCam, Cambridge, UK), rabbit IgG polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Genway, San Diego, CA, USA). Secondary antibody was IgG fraction monoclonal mouse anti-rabbit IgG, light chain specific, conjugated with HRP (Jackson ImmunoResearch, West Grove, PA, USA).

Sato defined medium was prepared as follows: DMEM (Invitrogen, Gaithersburg, MD, USA) supplemented with 25 μg/ml gentamicin (Invitrogen), 5 μg/ml bovine insulin, 50 μg/ml human transferrin, 100 μg/ml bovine serum albumin (BSA) Fraction V, 66 ng/ml progesterone, 16 μg/ml putrescine, sodium selenite 5 ng/ml, 400 ng/ml triiodothyroxine (T3) (Biofluids Inc., Rockville, MD, USA) and 400 ng/ml thyroxine (T4).

Experimental Procedures

Isolation of OP Cells—Neonatal OP cells were prepared from postnatal day 2 rat brains, by a method detailed in Armstrong [15]. Approximately 7-10 days after plating, the OP cells and microglia were dislodged by shaking the flasks, followed by plating on non-coated tissue culture plastic for 25-40 min to allow the differential adhesion of microglial cells. The cell suspension was collected, concentrated and resuspended in Sato defined medium with high insulin [16].

Migration Assay—Cell migration was assayed using the agarose drop assay [17-19]. Cells were exposed to inhibitory reagents for 1-2 h prior to addition of PDGF-A. Where cells were exposed to PDGF-A for 120, 60 or 30 min, the supernatant was removed carefully, replaced with serum/PDGF-A free Sato medium for 3-5 min, after which the supernatant was removed and replaced with serum free/PDGF-A free Sato medium for the duration of the study. Cells migrate away from the drop in a corona (Fig. 1a). The distance between the edge of the drop and the leading edge of migrating cells within the corona is recorded on four sides of the drop. The leading edge was assessed as the cell body of the furthest cell that is not further than ¼ square from the next cell to discount outlying cells from the measurements [17]. Measurements are taken at 0, 90, 180, and 270° angles around the drop. Plates are always aligned in a uniform manner on the microscope prior to measuring. Migration away from the edge of the drop is measured using a calibrated eyepiece graticule in which the width of one square is equivalent to 162 μm, on four sides of the drop every 24 h for 3 days.

Proliferation Assay—Purified OP cells were prepared as for the migration assay and plated on PDL coated glass coverslips in 1% FBS in Sato medium at 25,000 cells per well. The cells were left to settle for 2 h at 37° after which time, serum free Sato's was added to the well to wash the serum off the cells. The supernatant was then replaced with Sato medium containing 10 ng/ml PDGF-A. After 30, 60, or 120 min, the supernatant was removed from the cells and the coverslips washed with serum/PDGF-A free Sato medium twice before being replaced with serum/PDGF-A free Sato medium for the duration of the study. BrdU (10 μM) was added to each well for the final 2 h of culture. Immunodetection of incorporated BrdU was detected as previously detailed [20]; monoclonal anti-BrdU antibody was visualized with donkey anti-mouse IgG (H + L) F(ab')2 FITC conjugate. Cells were counter stained with the nuclear stain, 4′,6-diamidino-2-phenylindole dihydrochloride:hydrate (DAPI) prior to mounting. Cell proliferation was assessed as the percentage of DAPI positive (+) OP that were also BrdU +. At least 100 cells per coverslip were counted with 3-4 coverslips per treatment using an Olympus IX70 inverted microscope. Images were acquired via an attached Spot II Camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA), with associated software. For each coverslip, 5 fields of view (at 10× magnification) were counted at random. Total cell numbers (assessed by DAPI staining), and BrdU positive cell numbers (assessed by fluorescent conjugated primary antibody) were recorded for each field of view. For each treatment 3-5

coverslips were prepared in 4-6 different experiments. Counts for each coverslip were meaned, and then the experiments were meaned to give the final data. The experiment was blinded prior to the coverslips being counted to avoid subjectivity.

Cell Survival Assay—Two methods were utilized to assess cell survival. Propidium iodide was used to label cells that had died because of both apoptosis and necrosis. Activation of caspase-3 was used to assay apoptotic cells. Propidium iodide is excluded from live cells. Purified OPs were plated as for the proliferation assay. After the initial overnight incubation, the medium was removed, and replaced with serum free Sato for 6 h. After which time Sato ± PDGF (10 ng/ml) ± inhibitor was added to the chambers, and the cells incubated for either 24 or 72 h. Propidium iodide (25 μg/ml) was added to each well for the final 10 min of culture. Cells were then fixed with 4% paraformaldehyde for 10 min at RT and counter stained DAPI. Cell viability was assessed as the percentage of DAPI + PI negative (-) OPs per coverslip. At least 100 cells per coverslip were counted, with 3-4 coverslips per treatment and 3 experiments per inhibitor. Cell counting used an Olympus IX70 inverted microscope. Images were acquired via an attached Spot II Camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA), with associated software. For analysis of apoptosis, OPs were plated onto glass coverslips, as for the proliferation assay. After the initial PDGF-A ± inhibitor exposure, cells were incubated in serum free Sato medium for 24-72 h. Cells were then fixed in 4% paraformaldehyde for 10 min at RT. The cells were washed twice with 1% saponin (Sigma) in PBS and blocked in 10% normal goat serum, prior to incubation in rabbit polyclonal anti-activated caspase-3 (BioVision Research Products, Mountain View, CA, USA) for 1 h at RT. After a further wash with 1% saponin in PBS, the primary antibody was visualized using TRITC conjugated goat-anti rabbit secondary antibody (Jackson ImmunoResearch). Cell counts and imaging were performed using a Zeiss Axio Imager Z1 inverted microscope, with Axiocam monochrome digital camera and software to allow capture of images for light and fluorescence microscopy. For each coverslip, 7 fields of view (at 10 × magnification) were counted at random. Total cell numbers (assessed by DAPI staining), and caspase-3 positive cell numbers (assessed by FITC conjugated primary antibody) were recorded for each field of view. For each treatment, 5 coverslips were prepared in 5 different experiments. Counts for each coverslip were meaned, and then the experiments were meaned to give the final data. The experiment was blinded prior to the coverslips being counted to avoid subjectivity.

Western Blot Analysis of Protein Expression—Western blot analysis of protein expression was performed on whole cell lysates prepared from purified OP cells treated with PDGF-A in the presence or absence of pharmacological inhibitors. OP cells were treated with pharmacological inhibitors for 30 min. PDGF-A was added for 30 min, and then Laemmli sample buffer was added [21], and the lysates were boiled for 5 min and stored at -80°C until analysed. For analysis of ERK phosphorylation after 24 h, cells were plated onto PDL coated 6-well culture plates (Greiner, Frankfurt, Germany) in Sato medium supplemented with 0.5% FBS. Two hours after plating the wells were flooded with serum-free Sato medium for a further 4 h (reducing the FBS concentration to 0.025%). After which, the pharmacological inhibitor was added to the wells at the specified concentration. Cells were returned to the incubator for 1-2 h, following which the PDGF-A was added to the wells. After a further 30 min, the medium was removed from the wells, the cells were washed with serum-free Sato medium, and serum-free Sato medium containing the pharmacological inhibitor alone was added to the cells for the following 24 h. Cells were lysed by washing with PBS followed by addition of Laemmli sample buffer. Samples were boiled for 4-5 min then stored at -80°C until analysed.

Samples (20-30 μg total protein) were resolved by SDS-PAGE (12% gels; 100 V for 2 h) and transferred to PVDF membranes (GE Health Care Worldwide) using a semidry electroblotter (Owl Separation Systems, Portsmouth, NH, USA). Blots were blocked with 5% skimmed milk in Tris-buffered saline containing 0.5% Tween-20 (TBS-T) for 1 h at room temperature. The

blot was incubated with an appropriate dilution of the primary antibody (pERK, 1:2,000; panERK, 1:40,000; GAPDH, 1:1,000) in 5% skim in TBS-T at 4°C overnight. Membranes were incubated with HRP-conjugated secondary antibody in TBS with 5% BSA, for one hour at room temperature. A luminol-HRP-chemiluminescence reaction using the Chemilucent ECL Detection System (Chemicon, Temecula, CA, USA) was used to detect the protein bands and membranes exposed to X-ray film. Densitometry readings were performed using a FluorChem 8900 scanner (Alpha Innotech, San Leandro, CA, USA) with Alpha Ease FC software, and relative density value (RDV) was calculated against the total ERK1/2 band density for each sample, then normalized to the control RDV of 0. GAPDH levels were analysed to assess equality of sample loading. Three different cell preps were assayed by Western Blot and the mean densities for each band calculated, normalized to zero and these values meaned.

Statistics—One-way analysis of variance was used to test differences between migration curves, followed by Dunnett's *t*-test post hoc analysis. Student *t*-test was used to test the difference between means of cell count experiments.

Results

Short Term Exposure to PDGF-A is Sufficient to Drive OP Migration for up to 72 h

To investigate the effect of PDGF-A on OP migration, we used an agarose drop assay [17] (Fig. 1a). Transient exposure to PDGF-A for 30 min at the start of the migration assay resulted in 917.0 ± 8.6 μm migration as compared to 973.3 ± 6.6 μm migration by cells continuously exposed to PDGF-A (Fig. 1b). Transient exposure to PDGF-A did not induce proliferation, with 30, 60 and 120 min exposure resulting in 15.84 ± 3.7%, 15.33 ± 2.1% and 19.23 ± 1.9% BrdU positive cells, respectively, as compared to 65.49 ± 2.6% BrdU positive cells in wells continuously exposed to 10 ng/ml PDGF-A for 24 h (Fig. 1c). To further confirm that this PDGF-induced migration measure was not skewed by proliferation of OP cells, the glutamate receptor antagonist alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) was used to inhibit OP proliferation [22]. In our study, 200 μM AMPA had no effect on the extent of migration (Fig. 2a). Further, in the wells with AMPA, there was a marked reduction in cell density with the same extent of migration, suggesting a reduction in proliferation during the course of the study. Inhibition of proliferation by AMPA was confirmed with the BrdU incorporation assay. AMPA significantly reduced PDGF induced proliferation of OP cells, from 68.54 ± 4.7% BrdU positive cells in the presence of 10 ng/ml PDGF to 34.15 ± 3.2% BrdU positive cells in the presence of 10 ng/ml PDGF + 200 μM AMPA ($n = 5$ with 4-5 replicates; $P > 0.005$) (Fig. 2b). Our finding is consistent with previously published data [22]. This confirms that observed PDGF induced OP migration cannot be due to proliferation of cells distributed away from areas of high cell density.

PDGF Withdrawal

Previous studies have indicated that PDGF withdrawal causes increased OP death [23]. We assessed the extent of cell death 72 h after 30 min exposure to PDGF, using caspase-3 activation and propidium iodide. Very few cells (15.8 ± 1.5%) were positive for activated caspase-3, as measured by immunoreactivity (Fig. 3a, b). Further, propidium iodide was used to assess a combination of necrotic and apoptotic cell death. Percentage total cell death was 21.6 ± 1.5%, 24.8 ± 1.4% and 21.4 ± 1.7% in control (untreated), continuous PDGF exposure, and 30 min PDGF exposure, respectively (Fig. 3b). Further, after 30 min exposure to PDGF-A, OP cells do not terminally differentiate upon PDGF withdrawal, (Fig. 3c, d). Indeed, 72 h after PDGF-A withdrawal, most of the OP cells are still expressing PDGFRα, a marker of early lineage OLs (Fig. 3c). Additionally, few OP cells differentiated during this assay based on immunostaining for O1, a cell surface galactolipid present on the membrane of differentiated

OL. Expression of O1 was assessed 72 h after withdrawal of PDGF, 22.6 ± 2.3% were O1 positive among cells counter stained with DAPI (Fig. 3d).

PI_3 kinase is not Involved in PDGF-A Induced OP Migration

To test whether PI_3 K mediates PDGF-A induced OP migration, we used the PI_3 K specific inhibitor LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopran-4-one) [24]. LY294002 had no effect on PDGF-A induced migration of OP cells in the agarose drop assay, except at a 10 μM concentration, which reduced migration of OP by 27.6% (Fig. 4a). However, at 10 μM, LY294002 reduced PDGF-A enhanced survival by 17.3% (to 75.13%, as compared to 88.13% in cells exposed to PDGF-A alone) ($P < 0.05$) (Fig. 4b). Cell death was assessed using propidium iodide uptake. Thus, the reduction of migration seen in the presence of 10 μM LY294002 is likely due to increased cell death.

Extracellular Signal-Related (ERK) Signaling in PDGF-A Initiation of OP Migration

Multiple extracellular regulated kinases (ERKs) (formerly mitogen-activated protein kinases (MAPKs)) are activated by tyrosine kinase receptors, such as the PDGF receptor, including $p42^{MAPK}$/ERK2, $p44^{MAPK}$/ERK1, Jun kinases (JNK1, 2 and 3), and $p38^{MAPK}$ [25]. ERKs, JNKs, and $p38^{MAPK}$ have each been shown within other cell systems to play a significant role in the regulation of migration via different intracellular pathways. A widely used inhibitor of ERK signaling is PD098059 (2′-amino-3′-methoxyflavone) [26]. PD098059 had no effect on PDGF-A induced migration when used at concentrations ranging from 0.05 to 10 μM (data not shown), which extends to well above the EC_{50} of PD098059 reported as 3 μM [27]. At 10 μM, PDGF-A induced migration was only reduced by 12%, to 743 ± 49 μm compared to 843 ± 64 μm in the presence of PDGF-A (Fig. 5a). PD098059 at 10 μM has been demonstrated to inhibit ERK signaling in other cell types [26,28]. The data obtained in this study confirms our previous finding that PD098059 did not inhibit PDGF-A induced OP migration [19]. PD098059 specifically inhibits activation of MEK-1 (MAPK kinase 1) but does not effect the phosphorylation and activation of ERKs by MEK-1 or MEK-2 that have been activated by raf [26]. To more thoroughly assess the contribution of ERK signaling we repeated these studies using U0126 (1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)-butadiene), which has a 100-fold higher potency than PD098059 in blocking ERK signaling and acts by selectively inhibiting MEK-1 and MEK-2 phosphorylation and activation of ERKs [27,29]. U0126 inhibited PDGF-A induced OP migration in a dose dependent manner. OP migration was reduced by 23.3% in the presence of 5.0 μM U0126 (from 878 ± 19 μm after transient exposure to PDGF-A to 673 ± 18 μm in the presence of U0126) and 10 μM U0126 reduced OP migration by 25.9% (from 878 ± 19 μm after transient exposure to PDGF-A to 650 ± 35 μm), both of which are significantly reduced compared to control migration ($n = 5$ with 4-6 replicates; $P < 0.05$) (Fig. 5b). The same concentrations of U0126 had no effect on PDGF-A stimulated cell survival (Fig. 5c). From this we concluded that PDGF stimulated OP migration is regulated through the ERK signaling pathway.

$p38^{MAPK}$ Pathway is not Involved in OP Migration

SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole) is an inhibitor of both JNK and $p38^{MAPK}$ [30,31]. Thirty minutes pretreatment of cells with SB203580 prior to the addition of PDGF-A had no significant effect on OP migration (Fig. 6a). At 20 μM, SB203580 appeared to inhibit migration of OP by approximately 60% (539.2 ± 17.8 μm as compared to 856.8 ± 15.7 μm). However, this may be the result of reduced cell survival. At 20 μM SB203580, cell survival is significantly reduced by 21% ($n = 5$ with 4-6 replicates; $P < 0.005$) (Fig. 6b). This data indicates that JNK and $p38^{MAPK}$ signaling is not likely to play a significant role in PDGF-A induced OP migration.

ERK Signaling in OP Migration via Cytoplasmic Phospholipase A_2 ($cPLA_2$) Feedback Loop

Phosphorylation of ERK2 ($p42^{MAPK}$) activates $cPLA_2$ [32]. The activation of $cPLA_2$ results in increased arachidonic acid (AA) release, which in turn, activates protein kinase C (PKC) [14]. AA activation of PKC leads to the phosphorylation of Raf, forming a positive feedback loop within the ERK cascade. We used arachidonyltrifluoromethyl ketone ($AACOCF_3$) to inhibit $cPLA_2$ activation [14]. At 10 μM as previously demonstrated to block $cPLA_2$ activation [14], $AACOCF_3$ reduced OP migration 27.5% at 72 h when stimulated from a 30 min exposure to PDGF-A, compared to controls (588 ± 71 μm in the presence of $AACOCF_3$ as compared to 811 ± 39 μm; $n = 5$ with 4-6 replicates; $P < 0.01$) (Fig. 7a). This 10 μM, $AACOCF_3$ treatment had no significant effect on the survival of OP cells (Fig. 7b). These data indicate that not only is ERK signaling involved in the regulation of PDGF induced OP migration, but that feedback via $cPLA_2$ is important for migration to continue in the absence of the PDGF ligand.

Maintained ERK Phosphorylation from Transient PDGF Exposure

Western blot analysis was performed to monitor ERK phosphorylation in OP cells in response to 30-min PDGF-A treatment with and without pharmacological compounds (Fig. 8). Control (untreated) cells showed low levels of ERK activation (Fig. 8a, lane 1).Twenty-four hours after 30 min exposure to PDGF-A levels of pERK are markedly increased (Fig. 8a, lane 2). The level of pERK remains increased for the following 72 h following 30-min exposure to PDGF-A (Fig. 8a, lanes 3-5). All densitometry values were normalized to the control (Fig. 8a lane 1) relative densitometry value (RDV) of zero. To confirm the activity of the pharmacological inhibitors used in this study, we pretreated OP with PD098059 (10 μM), U0126 (10 μM) or $AACOCF_3$ (10 μM) for 2 h prior to adding the PDGF-A (Fig. 8b). OP cells were harvested immediately after PDGF-A treatment as well as 24 h later. Again, RDVs were normalized to the control RDV of zero (Fig. 8b, Lane 1). OP cells treated with PDGF-A for 30 min have increased pERK when harvested immediately (Fig. 8b, Lane 2) and at 24 h later (Fig. 8b, Lane 3). PD098059 did not appear to affect levels of pERK when cells were harvested immediately after PDGF-A treatment (Fig. 8b, Lane 4). In contrast, U0126 markedly reduced the levels of pERK in OP cells harvested immediately after PDGF-A treatment (Fig. 8b, Lane 5), and kept the levels of pERK reduced in the cells for at least the following 24 h (Fig. 8b, Lane 6). $AACOCF_3$ showed an apparent reduction in the level of pERK immediately after PDGF-A treatment (Fig. 8b, Lane 7) and at 24 h later (Fig. 8b, Lane 8). The relative band densities of cells treated with PDGF and pharmacological inhibitors shows a clear increase in pERK following PDGF-A treatment, which is not blocked by the addition PD098059, but is markedly reduced in the presence of U0126. pERK levels are also markedly reduced in the presence of $AACOCF_3$ (Fig. 8c). Immunocytochemistry shows that phospho-ERK is still increased in OP, 72 h after exposure to PDGF-A for 30 min (Fig. 8d).

Discussion

PDGF-A is known to promote migration of OP cells in vitro [5,33,34]. To further investigate PDGF-A signaling in OP migration, we used an agarose drop migration assay [17] that has been used previously to investigate regulatory mechanisms involved in the migration of OP [18,19]. This agarose drop assay has the advantage of allowing continuous monitoring of the cells while manipulating the culture medium components, e.g. adding pharmacological inhibitors of PDGF signaling components, and also enabling assessment of the proliferation and survival of the OP cells that migrated during the assay. Cell migration is measured as the distance the cells have moved from the edge of the drop. Changes in migration velocity, or direction of migration are not assessed, therefore this assay may be measuring a combination of different changes in cell migration. However, the agarose drop assay can be used to quantify changes in the extent of cell migration resulting from exposure to different factors, over a period

of three days. By measuring the distance migrated over 72 h we are identifying a cumulative movement of cells away from the edge of the agarose drop.

Transient Exposure to PDGF-A Drives OP Migration for up to 72 h

In the current study we found that OP cells exposed to PDGF-A for 30 min at the start of the migration assay migrated as far as cells exposed to PDGF-A continuously throughout the time period of the assay (3 days). However, there was a lower cell density in the wells exposed to PDGF-A for only 30 min. Several studies have shown that duration of receptor occupancy is essential to regulate the different downstream signaling pathways linked to tyrosine kinase receptors [35,36]. Heldin and colleagues have shown that ligand/receptor binding of 8 h or more is required to activate cell cycle progression [37]. Data from our study concurs with these previous studies as OP cells exposed to 10 ng/ml PDGF-A for up to 2 h and fixed 24 h later did not have a higher index of BrdU incorporation as compared to cells that were not exposed to PDGF. Thus, transient PDGF-A exposure does not stimulate OP proliferation.

PDGF Withdrawal

To determine the effects of PDGF-A withdrawal after transient PDGF exposure, we used two complementary assays of cell death, the activation of caspase-3 and propidium iodide uptake. Caspase-3 is an important effector caspase that is activated by both intrinsic and extrinsic apoptotic pathways [38]. We show no increase in the activation of caspase-3 in OP cells up to 72 h after withdrawal of the PDGF-A. Previously published studies have shown that PDGF-A is required for cell survival [7,9,12] and is essential to inhibit both OP differentiation and cell death [23]. Our findings differ from these previous studies in two distinct ways, (1) after 30 min exposure to PDGF-A, OP cells do not die when PDGF-A is withdrawn. (2) After 30 min exposure to PDGF-A, OP cells do not terminally differentiate, as assessed by changes in OL marker expression. In our study, the majority of OP still expressed PDGFRα at 72 h after PDGF-A withdrawal, and only a few cells expressed O1.

A simple explanation for these significantly different results is that in previous studies PDGF-A has been used to increase cell numbers and establish the cultures prior to running assays. Another study showed that growth factor naïve OP do not die after PDGF withdrawal [39]. Similarly, in our hands, the purified OP are not exposed to PDGF-A prior to the initial 30 min exposure, i.e. have not been grown in the presence of PDGF-A prior to running the migration and proliferation assays described in this study. It is our hypothesis that short term exposure to PDGF-A activates only the migratory activity of OP. Accordingly, longer term exposure of 8 h or more is required for the activation of other intracellular pathways leading to proliferation, such as PI_3 K [4,40].

PDGFRα Activation of Intracellular Signal Transduction

PDGF-A ligand binding to the PDGFRα results in a series of intracellular signaling cascade events. Including the phospholipase C-γ, PI_3 K, the Src family of tyrosine kinases, and the MAPKs, including ERK, cascades [41]. We assessed the role of these pathways in the regulation of OP migration.

PI_3 K is not Involved in PDGF-A Induced OP Migration

Inhibition of PI_3 K using the specific inhibitor, LY294002 [24], had no effect on PDGF-A induced migration of OP in the agarose drop assay. We saw an apparent reduction in PDGF induced migration in the presence of 10 μM LY294002. However, we also observed an increase in cell death in the presence of 10 μM LY294002, which provides a simple explanation for the apparent decrease in migration. The concentration of 10 μM has previously been shown to reduce cell survival, with 75% survival of OP at 24 h post treatment in the presence of 5 μM

LY294004 and ~55% survival in 10 μM LY294004 [42]. Our data showing cell death at 10 μM is consistent with this previously published data. Vemuri and McMorris [42] clearly demonstrate a role for PI_3 K in cell survival, supported by the findings of Ebner et al. [12]. In their study, Ebner and colleagues showed a distinct role for PI_3 K in proliferation and survival of spinal cord OP [12].

Contrary to our conclusion, McKinnon et al. reported a role for PI_3 K in the regulation of OP migration [40]. They assessed the migration of OP away from spinal cord explants to show that PDGFRα defective cells could be rescued with transgenic PDGFRα. In their study, if the transgene was PI_3 K activation-defective, OP cells did not migrate. An explanation for these contradictory results may be that we only expose the cells to PDGF-A for 30 min, whereas McKinnon and colleagues used PDGF-A to maintain their cultures for several days prior to the assay, withdrawing PDGF-A preceding initiation of the experiment [40]. PDGF induces proliferation after 8 h exposure of the cells to the mitogen [37] and may also result in activation of other intracellular pathways that may alter the PI_3 K dependence. Therefore, it is possible that increased cell numbers account for the movement of cells away from the explants in the McKinnon studies.

ERK Regulates PDGF Induction of OP Migration

ERK cascades are at the heart of numerous networks controlling different cellular processes including proliferation, migration, cell survival and cell death. In a recent study, Kato et al. [43] showed that phosphorylated ERK appears in the spinal cord of chick embryos in a specific spatio-temporal pattern that matches that of OP. Using immunoreactivity to localize phosphorylated ERK1/2 in the developing chick spinal cord and dorsal root ganglia, Kato et al. showed that pERK1/2 is co-localized to Olig2 positive cells in embryonic day 7 chick spinal cords. Olig2 encodes a transcription factor that is essential for motor neuron and OL development [44,45]. Due to other circumstantial evidence relating to the location of the pERK1/2, Kato et al. [43] concluded that pERK1/2 plays a role in the regulation of OP migration.

In our study, 10 μM PD098059, an inhibitor of ERK signaling had no effect on PDGF-A induced migration. However, PD098059 is only a partial inhibitor of ERK1/2 mediated intracellular signaling [29]. U0126 is a more selective inhibitor of both ERK1 and ERK2, with a 100-fold higher potency than PD098059 [27,29]. In our study, U0126 inhibited PDGF-A induced OP migration in a dose-dependent manner without affecting cell survival. This data indicates that PDGF-A induced OP migration is regulated via the ERK signaling pathway.

$p38^{MAPK}$ Pathway is not Involved in OP Migration

Previous studies have shown that $p42^{MAPK}$ is the pre-dominant MAPK isoform activated in response to PDGF-A binding [46]. There are, however, numerous different MAPK isoforms, including ERK5, JNK2 and $p38^{MAPK}$. As stated above, the presence of SB203580, which inhibits the activity of $p38^{MAPK}$ [30] did not inhibit PDGF-A induced OP migration in this study. This data indicates that $p38^{MAPK}$ does not play a significant role in regulating PDGF-A induced migration of OP.

Positive Feedback Loops in the ERK Signaling Cascade Maintain Signaling

The simple statement that PDGF-A induced OP migration is regulated via the ERK signaling pathway does not address how the activation of the ERK1/2 pathway can result in migration for up to 72 h when the PDGF-A stimulus is removed after only 30 min. We, therefore, investigated the complete ERK1/2 signaling pathway more closely. There are positive feedback loops within the ERK cascade that may play a role in the maintained signaling seen after transient activation of the receptor. Phosphorylation of ERK2 ($p42^{MAPK}$) activates $cPLA_2$

[33]. The activation of $cPLA_2$ results in increased AA release, which in turn, activates PKC [14,47]. PKC stimulates the ERK pathway through activation of Raf [14]. Bhalla et al. [14] provided strong evidence for this positive feedback loop in the ERK signaling pathway by using the specific $cPLA_2$ inhibitor $AACOCF_3$ [48]. In our study, 10 μM $AACOCF_3$ partially inhibited OP migration in the agarose drop assay, without affecting cell survival. These data indicate that the $cPLA_2$ signaling loop plays a role in maintaining the migration signal after short-term exposure to PDGF-A, but is not the sole regulatory mechanism. cPLA2 activation of arachidonic acid also leads to leukotriene and prostaglandin activation, which in turn may alter the cell cytoskeleton. Further experiments are required to assess the exact role of $cPLA_2$ in the maintenance of ERK activation leading to OP migration seen in this study.

Our study used a defined medium for maintenance of the cells, one that has been used previously in many studies [16]. This includes a relatively high concentration of insulin, the receptor for which is also a tyrosine kinase receptor. Therefore, it is possible that activation of the insulin growth factor is playing a role in the maintenance of the signaling pathways activated by PDGFRα activation.

Conclusion

In the present study, we observed that OP cells stimulated with PDGF-A for a 30-min period subsequently continued active migration in the absence of continued exposure to PDGF-A. This migration of OP cells in response to transient exposure to PDGF-A is equivalent to the migration measured in the presence of continuous PDGF-A, but without an increase in cell numbers as occurs with continuous PDGF-A treatment. We establish that a 30-min pulse of PDGF-A is sufficient to activate the ERK signaling pathway. Activation of a positive feedback loop, which utilizes $cPLA_2$ to maintain ERK phosphorylation, helps maintain OP cells in a migratory phase for at least the following 72 h. The data presented in this study provide evidence to support the hypothesis that PDGF-A acts to initiate OP migration during early CNS development.

Acknowledgements

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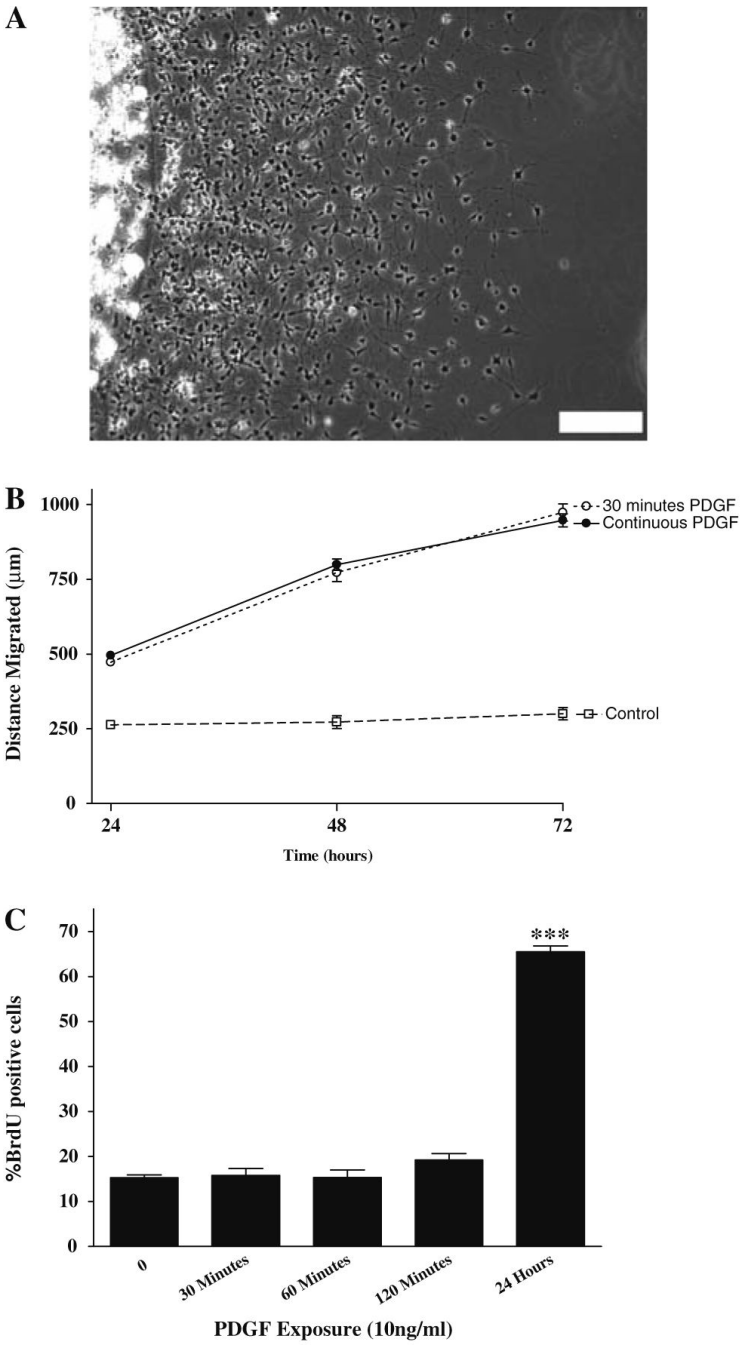

Fig. 1.

Transient PDGF-A exposure induces OP migration without affecting proliferation. (**a**) Phase contrast photomicrograph showing PDGF-A (10 ng/ml) inducted OP migration away from the edge of the agarose drop (bar = 0.1 mm). (**b**) After 30 min (open circles/dashed line) of PDGF-A (10 ng/ml) exposure, OP cells migrate to the same extent as OP cells continuously exposed to PDGF-A (closed circles). Control OP cells (no PDGF; open squares/dashed line) do not migrate away from the drop. (**c**) Transient exposure to PDGF-A (10 ng/ml) of up to 120 min has no effect on OP proliferation. $n = 5$ with 4-6 replicates ***$P < 0.005$ as compared to untreated cells

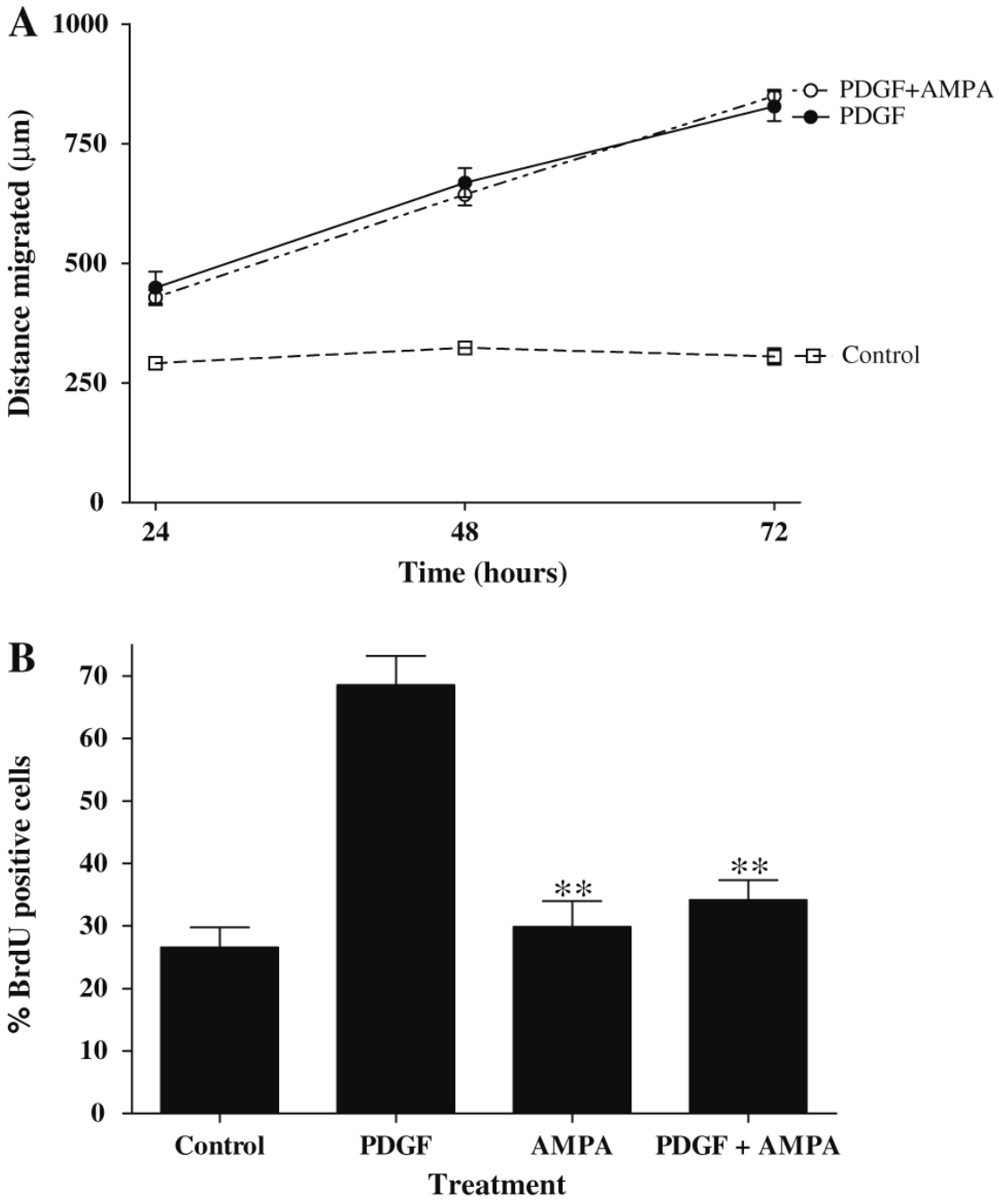

Fig. 2.

Inhibiting OP cell proliferation with AMPA does not effect migration stimulated by PDGF-A. (**a**) The glutamate receptor agonist AMPA has no effect on OP migration in response to PDGF-A (10 ng/ml) exposure: Control (no PDGF)—open squares/dashed line; PDGF-A (10 ng/ml)—closed circles; 10 ng/ml PDGF-A + 200 μM AMPA—open circles/dashed line. Vertical bar represents sem, $n = 5$ with 4-6 replicates. (**b**) 200 μM AMPA significantly reduces OP proliferation in response to 10 ng/ml PDGF-A. Counts taken 48 h after treatment with PDGF-A and or AMPA. Vertical bar represents sem. $n = 5$ with 4-6 replicates. $^{**}P < 0.01$ as compared to cells exposed to PDGF by Student *t*-test

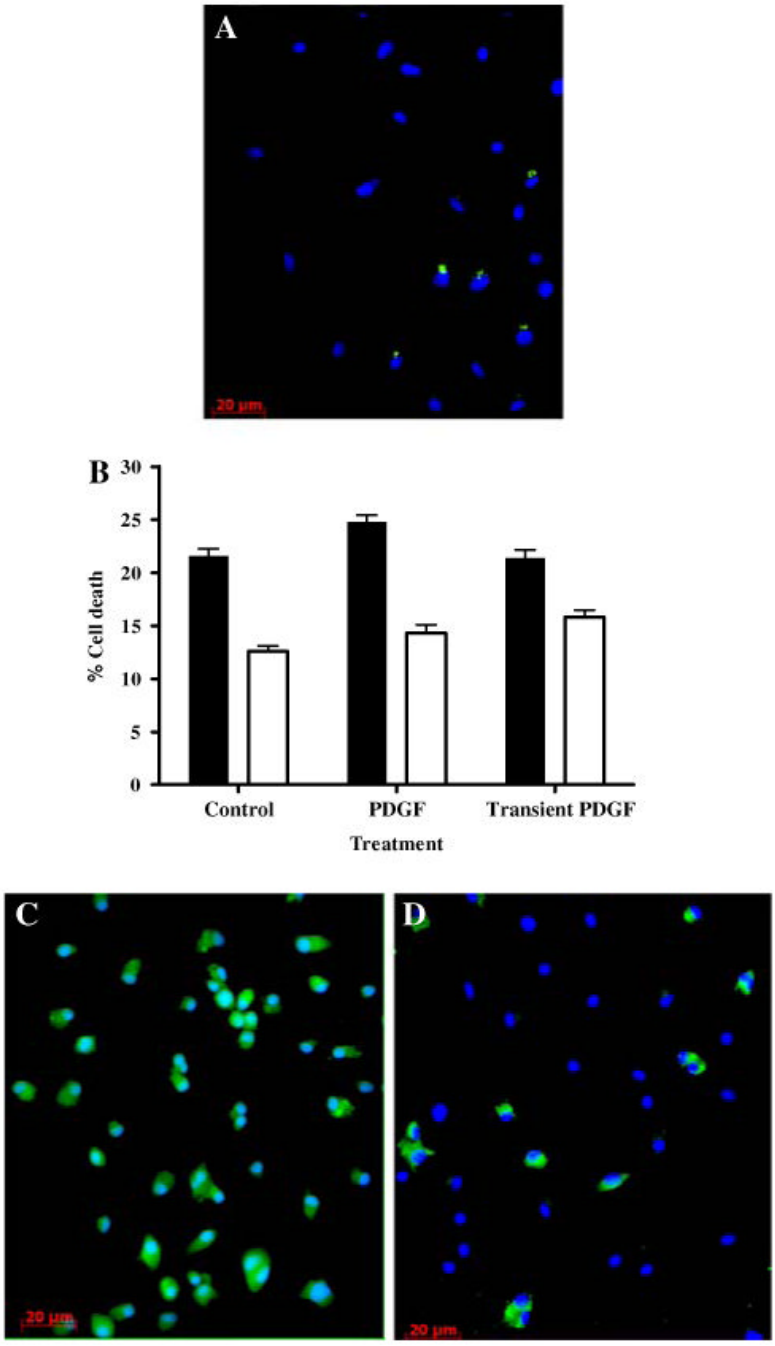

Fig. 3.

Transient PDGF-A exposure does not affect cell survival or differentiation. (**a**) Immunocytochemistry to detect activated caspase-3 in OP cells exposed to 10 ng/ml PDGF-A for 30 min reveals no significant increase in cell death after 72 h (Blue = DAPI; Green = activated caspase-3). (**b**) Transient PDGF-A (10 ng/ml) exposure does not result in increased cell necrosis in OP, as assessed by propidium iodide uptake 24 h after treatment with PDGF-A (10 ng/ml) (black bars) or increased apoptosis, as assessed by caspase-3 activation (white bars) (*n* = 5). OP cells treated for 30 min with PDGF-A (10 ng/ml) remain predominantly PDGFRα positive (**c**) (Blue = DAPI; Green = PDGFRα), with only a few cells expressing O1

(**d**) (Blue = DAPI; Green = O1), indicating that OP cells do not differentiate within the duration of the assay (72 h)

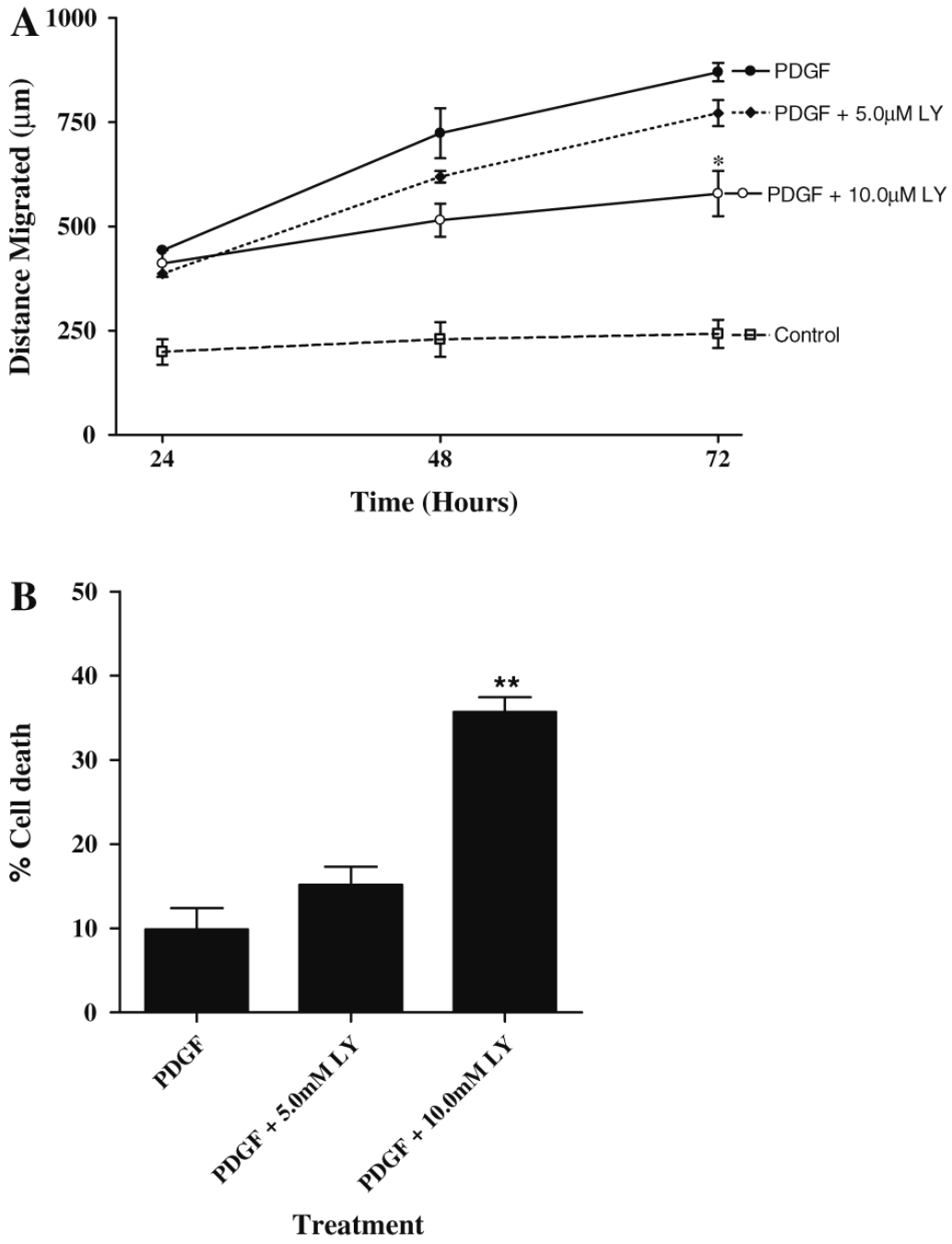

Fig. 4.

PI_3 K pathway is not involved in OP migration induced by transient exposure to PDGF-A. (**a**) The PI_3 K specific inhibitor LY294002 has no effect on PDGF-A induced OP migration at 5 μM (closed diamond, dashed line). However, at 10 μM, LY294002 significantly reduces PDGF-A (10 ng/ml) induced OP migration (open circles), compared to OP migration induced by 30-min PDGF-A exposure (10 ng/ml) (closed circles), *n* = 5 with 4-6 replicates. (**b**) At 10 μM, LY294002 significantly increases cell apoptosis, as assessed by caspase-3 activation after 72 h, indicating that the reduced migration seen in the presence of this inhibitor may be due to increased cell death, rather than direct inhibition of migration (*n* = 3). Vertical bar represents

sem. *$P < 0.05$, ***$P < 0.005$ as compared with continuous PDGF-A (10 ng/ml) exposure by Student *t*-test

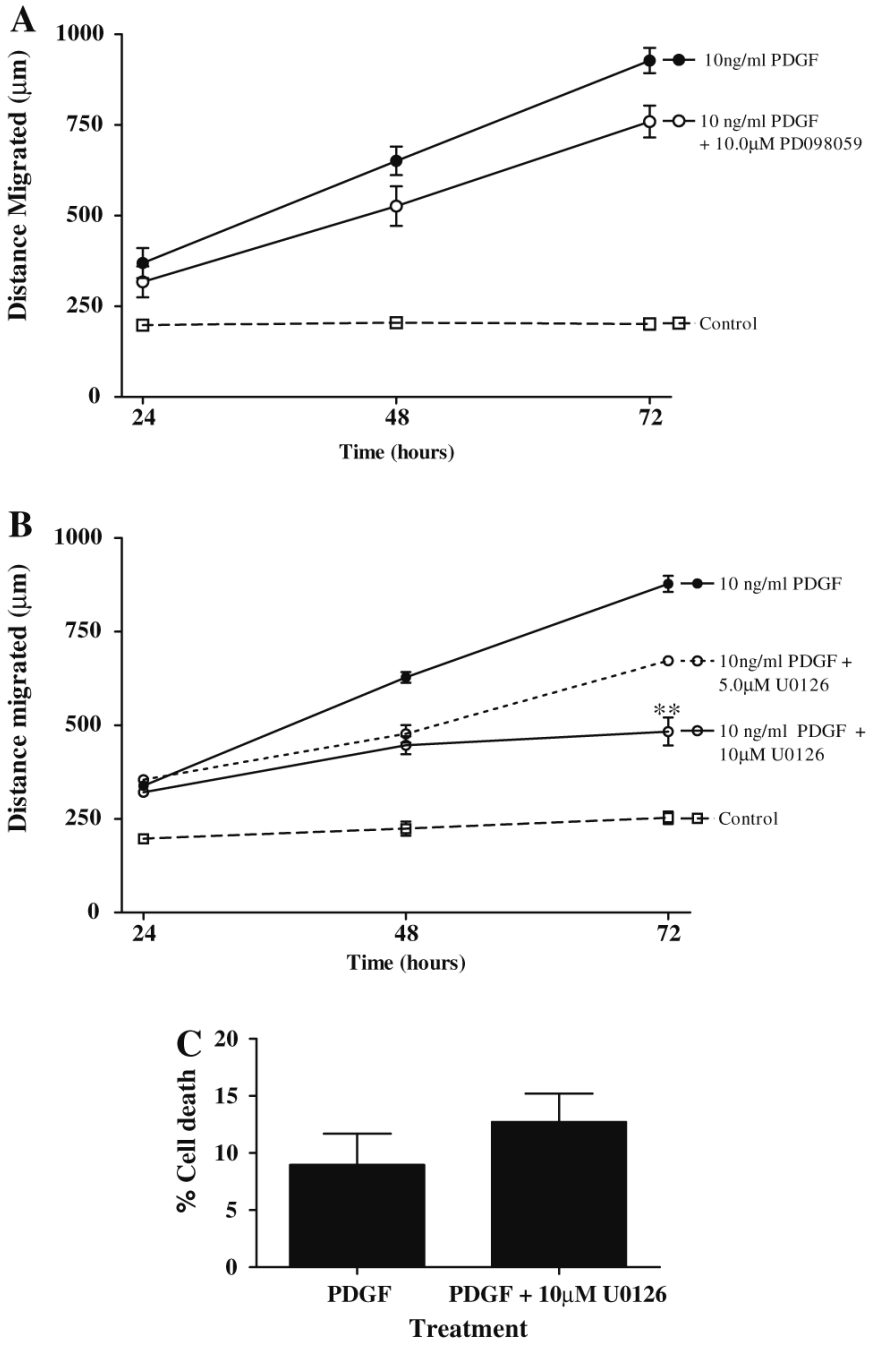

Fig. 5.

The role of ERKs in the migration of OP after 30-min exposure to 10 ng/ml PDGF-A. (**a**) At 10 μM, PD098059, an inhibitor of MEK1 activation, had no significant effect on PDGF-A (10 ng/ml) induced OP migration over 72 h (open circles with PD089059; closed circles, without compound). Cells do not migrate significant distances in the absence of PDGF (open squares/dashed line) (*n* = 5 with 4-6 replicates). (**b**) U0126 inhibition of MEK1 MEK2 significantly reduced PDGF-A (10 ng/ml) induced OP migration at both 5 μM (open circles/dashed line) and 10 μM (open circles) concentrations (*n* = 7 with 4-6 replicates). (**c**) At 10 μM, U0126 had no significant effect on OP survival. *n* = 5 with 3 replicates. Vertical bar represents sem. **P* <

0.05, **$P < 0.01$, ***$P < 0.005$ as compared with continuous PDGF-A exposure by Students *t*-test

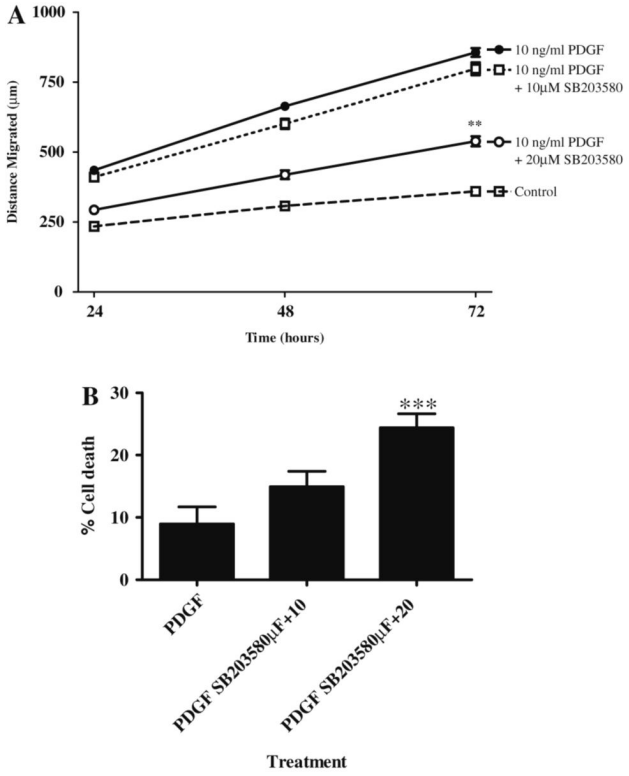

Fig. 6.

The p38^{MAPK} is not involved in regulating OP migration. (**a**) The p38^{MAPK} specific inhibitor SB203580 had no effect on PDGF-A induced OP migration at 10 μM (open boxes, dashed line). However, at 20 μM, PDGF-A induced OP migration is significantly inhibited (open circles with SB203580; closed circles without compound) (n = 5 with 4-6 replicates). (**b**) Propidium iodide assessment of OP cell death in the presence of 20 μM SB203580, shows significant inhibition of PDGF-A enhanced survival of OP cells after 24 h, indicating that the reduced level of migration measured at this concentration is due to induction of cell death rather than an inhibition of active migration. n = 5 with 3 replicates. Vertical bar represents sem. *P < 0.05, **P < 0.01, ***P < 0.005 as compared with continuous PDGF-A exposure by Students t-test

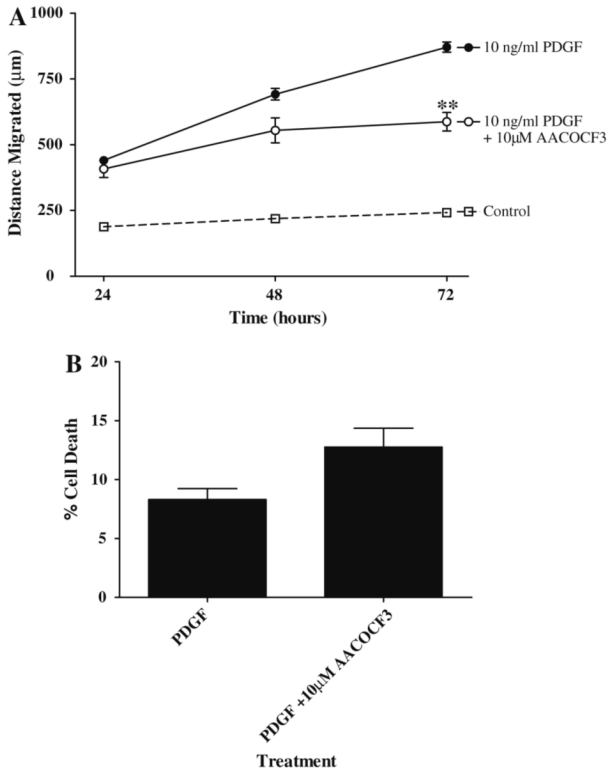

Fig. 7.

Cytoplasmic phospholipase$_2$ (cPLA$_2$) acts to maintain OP migration induced by transient exposure to 10 ng/ml PDGF-A. (**a**) Inhibition of cPLA$_2$ using the selective inhibitor AACOCF$_3$ significantly reduced PDGF-A induced OP migration at 10 μM (open circles). The inhibition was not complete, indicating that the positive feedback loop regulated by cPLA$_2$ is not the sole regulator of PDGF-A (10 ng/ml) induced OP migration. Control (untreated) OP do not migrate significant distances from the edge of the drop (open squares/dashed line) (n = 5 with 4-6 replicates). (**b**) At 10 μM, the cPLA$_2$ inhibitor, AACOCF$_3$ had no significant effect on OP death measured by caspase-3 activation. Therefore, the reduction in migration is not due to cell death induced by inhibition of the cPLA$_2$ signaling (n = 3). Vertical bar represents sem. $^{**}P < 0.005$ as compared with continuous PDGF-A exposure by Student t-test

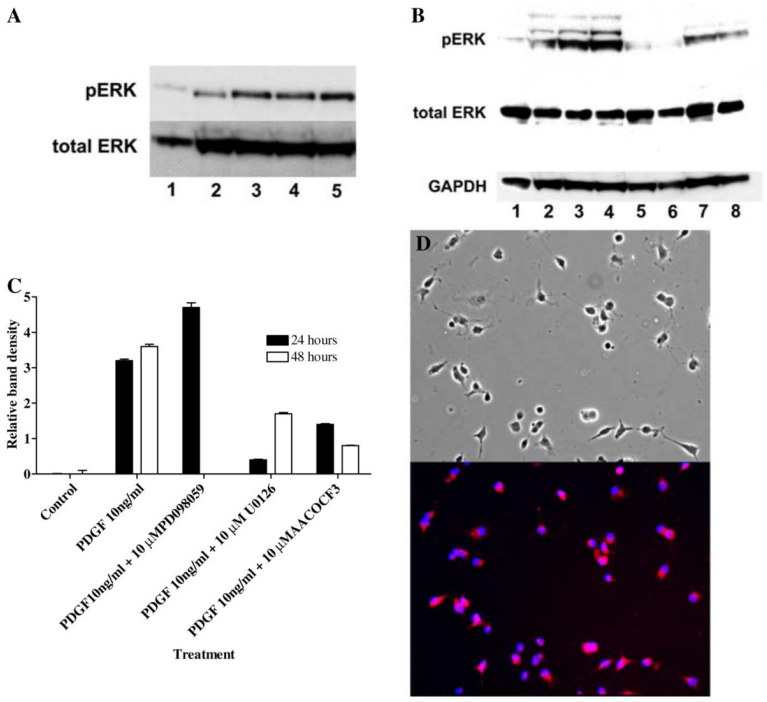

Fig. 8.

Western blot analysis of ERK phosphorylation. (**a**) Western blot analysis of ERK2 phosphorylation in OP cells. Lane 1—Control OP cells (no PDGF). Lane 2: Cells harvested 24 h after 30 min treatment with 10 ng/ml PDGF-A. Lanes 3-5: Replicates of cells harvested 72 h after 30 min treatment with 10 ng/ml PDGF-A. (**b**) Western blot analysis of ERK phosphorylation after 30-min PDGF-A treatment in the presence of pharmacological inhibitors. Lane 1: Control OP cells (no PDGF). Lane 2: OP cells harvested immediately after 30-min 10 ng/ml PDGF-A treatment. Lane 3: OP cells harvested 24 h after 30-min PDGF-A treatment. Lane 4: OP cells treated for 30 min with PD098059 prior to addition of PDGF-A for 30 min then harvested immediately. Lane 5: OP cells treated for 30 min with U0126 prior to addition of 10 ng/ml PDGF-A for 30 min then harvested immediately. Lane 6: OP cells treated for 30 min with U0126 prior to the addition of PDGF for 30 min and harvested 24 h later. Lane 7: OP cells treated for 30 min with $AACOCF_3$ prior to the addition of PDGF-A for 30 min then harvested immediately. Lane 8: OP cells treated for 30 min with $AACOCF_3$ prior to the addition of 10 ng/ml PDGF-A for 30 min and harvested 24 h later. (**c**) Relative density values for the phosphoERK protein bands obtained by Western Blot. Values were normalized to the control (no PDGF-A treatment) value, and data is shown for cells lysed 24 h (black bars) and 48 h (white bars) after treatment. (**d**) Immunocytochemistry of OP 72 h after transient exposure to PDGF-A shows phospho-ERK in the majority of cells migrating away from an agarose drop