PDGF-induced signaling in proliferating and differentiated vascular smooth muscle: Effects of altered intracellular Ca\(^{2+}\) regulation

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Abstract

Objective: Platelet-derived growth factor-BB (PDGF)-induced intracellular signaling is involved in phenotypic modulation of vascular smooth muscle (VSM). This study has examined the PDGF-induced Ca\(^{2+}\) increase and the resultant effect on signaling pathways in proliferative compared with fully differentiated VSM.

Methods: PDGF-induced changes in Ca\(^{2+}\) were measured in portal vein (PV) myocytes from 2–4-day-old (proliferating), compared to 6-week-old (differentiated), Sprague Dawley rats. Phospholipase C (PLC)\(^\gamma\) expression and activation of extracellular signal-regulated kinase (ERK) 1/2 was determined by immunoblotting or confocal immunolabelling. Activation of the Ca\(^{2+}\)-dependent transcription factor, nuclear factor of activated T-cells (NFATc), was assessed by electromobility shift assay.

Results: PDGF increased the intracellular Ca\(^{2+}\) concentration in differentiated, but not in proliferating, PV myocytes. This is probably due to very low expression of PLC\(^\gamma\) in proliferating PV. In 6-week-old PV, PDGF stimulation induced nuclear translocation and activation of NFATc. PDGF did not induce NFATc activation in neonatal PV. PDGF-induced ERK1/2 activation was observed in both 2–4-day-old and 6-week-old PV. In 6-week-old PV, ERK1/2 activation was Ca\(^{2+}\)-dependent and protein kinase C-dependent. However in 2–4-day-old PV, PDGF-induced ERK1/2 activation was via a Ca\(^{2+}\)-independent, atypical protein kinase C. PLC\(^\gamma\) expression was also decreased in the neointima, compared to media, of balloon-injured rabbit subclavian arteries.

Conclusions: The regulation of PDGF-induced Ca\(^{2+}\) increases by PLC\(^\gamma\) expression in VSM may provide a mechanism for coordinating different signaling pathways leading to activation of specific transcription factors. This may play an important role in the phenotypic modulation of VSM.

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1. Introduction

In vascular disease, a phenotypic modulation of vascular smooth muscle (VSM) cells occurs from a differentiated contractile phenotype to a proliferative phenotype [1,2]. This is the result of mitogenic stimuli that produce a change in gene expression. Studies of VSM proliferation have focussed on growth factors such as platelet-derived growth factor-BB (PDGF). PDGF stimulates mitogenic pathways, leading to the activation of transcription factors required for VSM proliferation [3]. PDGF activation in VSM cells is principally associated with mitogenic responses via the PDGF\(\beta\) receptor, a tyrosine kinase receptor which undergoes autophosphorylation leading to the initiation of a variety of intracellular pathways [4]. One such pathway is the activation of phospholipase C (PLC)\(\gamma\). PLC\(\gamma\) activation leads to the formation of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (InsP\(_3\)) with an associated intracellular
Ca\(^{2+}\) release [5]. In addition, PDGF\(\beta\) receptor signaling also results in extracellular signal-regulated (ERK)1/2 activation in VSM [6,7]. This can occur via protein kinase C activation [7], as a result of PLC\(\gamma\)-induced DAG formation. Therefore, PLC\(\gamma\) activation could have a pivotal role in regulating PDGF-induced mitogenic responses mediated by either increases in [Ca\(^{2+}\)], or by protein kinase C and subsequent ERK1/2 activation.

Both [Ca\(^{2+}\)], and ERK1/2 are known to have important roles in transcription factor activation. In VSM cells, ERK1/2 activation regulates early growth response genes necessary for VSM proliferation and is increased in experimental models of vascular injury [8]. In addition, transfection of dominant-negative ERK1/2 prevents neointimal formation in balloon-injured arteries [9]. It is also now established that changes in intracellular Ca\(^{2+}\) can regulate different transcription factors involved in VSM growth signals [10]. For example, in native VSM, the Ca\(^{2+}\)-dependent transcription factor family, NFATc (associated with VSM proliferation [11]) is activated by InsP\(_3\)-induced Ca\(^{2+}\) release from intracellular stores [12]. Regulation of Ca\(^{2+}\) responses, with respect to transcription factors, may occur via different agonists/stimuli or possibly via alterations in Ca\(^{2+}\) channel expression [12–15].

In the present study we have investigated the PDGF-induced [Ca\(^{2+}\)], increase in proliferating and fully differentiated VSM. PDGF produced a rise in intracellular Ca\(^{2+}\) in fully differentiated portal vein and this was followed by increased activation of the Ca\(^{2+}\)-dependent transcription factor NFATc. However, in proliferating portal vein myocytes no Ca\(^{2+}\) rise was observed following PDGF stimulation. In addition, PDGF did not activate NFATc in proliferating portal vein, possibly due in part to significantly lower PLC\(\gamma\) expression in proliferating VSM compared to differentiated VSM. The differential Ca\(^{2+}\) homeostasis resulted in other changes to PDGF-induced intracellular signaling pathways. PDGF-induced ERK1/2 activation was observed in both VSM phenotypes, although this was Ca\(^{2+}\)-independent in neonatal portal vein and Ca\(^{2+}\)-dependent in differentiated portal vein. Therefore, the PDGF-induced Ca\(^{2+}\) homeostasis may be dependent upon the growth state of VSM cells and controlled by expression of PLC\(\gamma\). This may provide a mechanism that allows differential regulation of transcription factors and growth signals by PDGF.

2. Methods

2.1. Portal vein preparation

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Male Sprague Dawley rats (6-weeks-old, 300–350 g) were euthanized by inhalation of CO\(_2\) followed by cervical dislocation, neonatal male Sprague Dawley rats (2–4-days-old, 6–10 g) were killed by stunning followed by cervical dislocation, and the portal vein was immediately removed. All procedures were in accordance with institutional guidelines. Veins were placed into ice-cold HEPES-buffered Krebs solution (in mmol/L: 137 NaCl, 5.9 KCl, 1.2 MgCl\(_2\), 1.2 CaCl\(_2\), 11 glucose, 11.6 HEPES, pH 7.4) cleaned of connective tissue and the endothelium was removed by gentle rubbing of the lumen [15]. Immunocytochemical and electron microscopy studies have shown that the portal vein consists predominantly of smooth muscle cells (data not shown).

2.2. Enzymatic dissociation of VSM myocytes

Smooth muscle cells from adult and neonatal portal vein were isolated by enzymatic dissociation as described previously [16]. Isolated smooth muscle cells were stored on glass-bottom dishes maintained at 4 °C and used within 6 h. Only cells that had an elongated morphology, i.e. relaxed smooth muscle cells, were used for imaging.

2.3. Imaging of [Ca\(^{2+}\)]

Individual VSM myocytes were loaded with 2 \(\mu\)mol/L Fura-2 AM for 40 min in Krebs buffer (in mmol/L: 130 NaCl, 5.6 KCl, 1.7 CaCl\(_2\), 11 glucose, 10 HEPES, pH 7.4) followed by a 20 min de-esterification period. A Zeiss Axiovert 200 inverted microscope, equipped with a cooled CCD camera (Photometrics) and a polychromatic illumination system (T.I.L.L. Photonics), was used to capture fluorescence images with excitations at 340 and 380 nm. The ratio of the fluorescence intensity between the pair of frames (FR340/380) was calculated after background subtraction. The Metafluor 4.6 software (Universal Imaging Corporation) controlled the illuminator and camera, and performed image ratioing and analysis. Results are expressed as F340/380 ratio. Experiments were carried out at room temperature (22–24 °C).

2.4. Immunoblotting

Dissected portal veins were pre-incubated at 37 °C for 45 min which gave a consistently low baseline level of activation for all proteins assessed. Following initial isolation of blood vessels, care was taken to avoid shear stress. All agonists and inhibitors were added to solutions already containing the isolated, preincubated blood vessels. The concentrations of inhibitors and drugs used were taken from previous studies and the specificity based on manufacturer’s instructions. Inhibitors, if required, were added for a further 30 min, following which portal veins were stimulated with PDGF (50 ng/mL). For PMA downregulation experiments, freshly isolated portal veins were maintained in Dulbecco’s modified Eagles medium without serum. The tissues were added to lysis buffer and homogenized at 4 °C in a Braun homogenizing vessel.
For neonatal portal veins, the tissue harvested from at least 2 neonatal rats was pooled in order to obtain sufficient protein. Protein was measured using a Lowry protein assay to ensure equal protein loading. In addition, samples were checked using Coomassie Blue stained gels, and membranes were stained with Ponceau Red to confirm equal protein loading. Whole-tissue homogenates were used for immunoblotting with anti-ERK1/2 antibodies (phospho-and pan-). Membrane preparations were used for PDGFβ receptor immunoblots and NFATc. Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane as previously described [16]. The membranes were immunoblotted with primary antibodies and immunoreactive bands were visualized using enhanced chemiluminescence quantified with an imaging densitometer (Biorad GS-690). Only blots that were entirely non-saturated pixels (as determined using Multianalyst software, Biorad) were quantified to ensure linearity of densitometric analysis.

2.5. Electrophoretic-mobility shift assays

Portal vein were stimulated with PDGF (50 ng/mL) for 30 min. Nuclear fractions were prepared according to the method by Dignam et al. [17]. Protein (10 µg) from nuclear preparations was used in each experiment. Biotin-labelled consensus oligonucleotides for the transcription factor NFAT (5′-ACGCACAAAGAGAAAATTTGTTCATACA) were incubated with nuclear fractions according to the manufacturer’s instructions (Panomics, USA). For competition experiments, unlabelled probe was added in addition to the biotin-labelled probe. Protein–DNA complexes were resolved on a 6% polyacrylamide gel. Bands were visualized using a streptavidin–HRP detection reagent as per manufacturer’s instructions (Panomics, USA).

2.6. In vivo balloon angioplasty

Procedures were performed as previously described and characterized [18]. New Zealand white rabbits were sedated by an intramuscular injection of Hypnorm® (fluanisone–fentanyl citrate mixture) and anesthesia was induced and maintained with a mixture of 2% nitrous oxide and 1.5–2% halothane in oxygen. The left femoral artery was exposed and a 3.0 mm balloon angioplasty catheter (Advanced Cardiovascular Systems Temecula, CA, USA) introduced into the subclavian artery. The balloon was inflated twice to 10 atm for 30 s. A third inflation to 8 atm was performed and the balloon was withdrawn by half its own length to ensure endothelial damage. All surgical procedures were performed under a Project License issued under the UK Home Office Animals (Scientific Procedures) Act 1986 (PPL 60/1988). Animals were euthanized 28 days after angioplasty and the subclavian arteries harvested.

2.7. Immunofluorescence

Arteries were fixed and frozen as previously described [15] and stained with primary antibody (either mouse anti-PLCγ1 or anti-smooth muscle actin antibody) followed by secondary antibody (FITC anti-mouse IgG in 2% BSA/PBS). BOBO-3 (1:1000 in PBS, Molecular probes) was used for identification of nuclei. Immunofluorescence was detected using a Biorad 1024 laser scanning confocal microscope. Specificity of immune staining was confirmed by the absence of fluorescence in sections incubated with mouse non-immune serum instead of primary antibody.

2.8. Analysis of data

Data are expressed as mean±SEM. Significance was tested by means of Student’s t test or ANOVA where appropriate. P<0.05 was considered significant.

3. Results

3.1. PDGF-induced increase in [Ca²⁺], and expression of PLCγ

In portal vein myocytes from 6-week-old rats, application of PDGF elicited an increase in the [Ca²⁺]. This consisted of an initial transient which, in more than 90% of myocytes, was followed by regular Ca²⁺ oscillations (Fig. 1A). PDGF was unable to produce any change in the [Ca²⁺], in myocytes from 2- to 4-day-old portal vein (Fig. 1A). However, stimulation with endothelin-1 produced a significant rise in the [Ca²⁺], (not shown) as previously demonstrated [19]. In parallel with Ca²⁺ imaging experiments, expression of both isoforms of PLCγ (PLCγ1 and PLCγ2) and PDGFβ receptor expression were compared at the same developmental stage. PDGFβ receptor expression was similar in 2–4-day-old portal vein, compared to 6-week-old portal vein (Fig. 1A). However, both PLCγ1 and PLCγ2 were expressed at very low levels in 2–4-day-old portal vein compared to fully differentiated portal vein (Fig. 1A).

The expression of PLCγ1 was determined during portal vein development. Several developmental timepoints (based on previous studies) were examined from 2-day-old (neonatal) to 6-week-old (fully differentiated) portal vein (Fig. 1B). At 2-day-old, levels of PLCγ expression were close to detection limits. At later postnatal developmental stages, expression of PLCγ increased reaching a maximum at 4–6 weeks. Concomitantly, the expression of PCNA, a marker of proliferating cells, decreased from 2-day-old reaching the minimum level at 6-week-old. In contrast, calponin (a marker of differentiated VSM) increased throughout the developmental stages examined, reaching a maximum at between 4 and 6 weeks old.
3.2. PDGF-induced NFATc activation

PDGF-induced activation of the Ca\(^{2+}\)-dependent transcription factor, NFATc, in both VSM phenotypes was measured. For measurement of nuclear translocation, portal vein was stimulated for 30 min with PDGF. Nuclear and cytoplasmic fractions were subjected to immunoblotting with anti-NFATc3 antibody. This isoform is predominantly expressed in at least some VSM types\[12,13\], and...
clearly detectable in portal vein. In neonatal portal vein, there was no change in the translocation of NFATc3 following PDGF stimulation (Fig. 2A). However, PDGF produced a significant translocation of NFATc3 to the nucleus in 6-week-old portal vein, and a concurrent decrease in cytoplasmic NFATc3. To determine whether an increase in \([\text{Ca}^{2+}]_i\), could result in NFATc translocation, neonatal portal veins were incubated with thapsigargin for 15 min. This resulted in a significant translocation of NFATc3 from the cytoplasm to the nucleus (Fig 2B). EMSA was used to support the results from immunoblotting experiments (Fig. 2B). A shift, denoting NFATc binding to the sequence-specific NFAT consensus oligonucleotide, was observed only in the nuclear fractions of fully differentiated portal vein following PDGF stimulation, but not in the nuclear fractions from neonatal portal vein. Addition of cold probe to the reaction mix abolished the PDGF-induced shift.

### 3.3. PDGF-induced ERK1/2 activation: role of PLC and \([\text{Ca}^{2+}]_i\)

PDGF was able to induce an increased phosphorylation of ERK1/2 in both neonatal and fully developed portal vein (Fig. 3). A 15-min incubation timepoint was chosen based on the timecourse for PDGF-induced phosphorylation of ERK1/2 (data not shown).

In order to determine the role of PLC in ERK1/2 activation, PDGF-induced ERK1/2 phosphorylation was measured in the presence of the selective PLC inhibitor, U73122. This inhibitor had no effect on PDGF-induced ERK1/2 phosphorylation in 2–4-day-old portal vein but significantly inhibited ERK1/2 activation in 6-week-old portal vein (Fig. 3A). The inactive inhibitor analogue, U73433 had no effect (not shown). To determine the role of the \([\text{Ca}^{2+}]_i\) in ERK1/2 activation, portal vein from neonatal or 6-week-old rats were pre-incubated with either BAPTA/AM or 2-APB (10 μmol/L) 15 min before PDGF stimulation.
(an intracellular Ca\(^{2+}\) chelator) or 2-APB (a partially selective inhibitor of InsP\(_3\)-induced Ca\(^{2+}\) release). 2-APB not only blocks InsP\(_3\) receptors and subsequent Ca\(^{2+}\) release from stores but also blocks store-operated Ca\(^{2+}\) entry activated in response to depletion of stores [20]. Pre-incubation with either BAPTA or 2-APB completely inhibited PDGF-induced ERK1/2 activation in 6-week fully differentiated portal vein (Fig. 3B). However, in 2–4-day-old portal vein, pre-incubation with either BAPTA or 2-APB had no effect on ERK1/2 activation induced by PDGF (Fig. 3B).

3.4. PDGF-induced ERK1/2 activation: role of protein kinase C isoforms

The potential involvement of protein kinase C isoforms in ERK1/2 activation was determined using selective protein kinase C inhibitors. Portal vein from 2–4-day-old and 6-week-old rats were preincubated with the broad spectrum protein kinase C inhibitor, Ro-31-8220, which inhibits conventional, novel and atypical protein kinase C isoforms. Ro-31-8220 preincubation completely inhibited PDGF-induced ERK1/2 phosphorylation in both neonatal and fully differentiated PV (Fig. 4A).

Preincubation with an inhibitor selective for Ca\(^{2+}\)-dependent (conventional) protein kinase C isoforms, Go\(_69776\), also significantly decreased PDGF-induced ERK1/2 activation in 6-week-old portal vein but had no effect on PDGF-induced ERK1/2 activation in 2–4-day-old portal vein (Fig. 4A). This suggests that in neonatal portal vein, PDGF activates ERK1/2 via a Ca\(^{2+}\)-independent protein kinase C isoform. In order to further determine the protein kinase C isoforms involved, portal vein were treated with 20

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**Fig. 4.** The role of protein kinase C in ERK1/2 activation was examined. (A) Pre-incubation with Ro-31-8220 (20 \(\mu\)mol/L), a general protein kinase C inhibitor or Go\(_69776\) (10 \(\mu\)mol/L), an inhibitor of Ca\(^{2+}\)-dependent protein kinase C isoforms, significantly decreased PDGF-induced ERK1/2 activation in 6-week-old portal vein (\(n=3\)). In neonatal portal vein, PDGF-induced ERK1/2 activation was also inhibited by preincubation with Ro-31-8220, but was unaffected by preincubation with Go\(_69776\) (\(n=3\)). (B) Immunoblots showing protein kinase C expression following downregulation of phorbol ester-sensitive protein kinase C (48 h with 20 \(\mu\)mol/L PMA). This prolonged incubation did not affect expression of the atypical protein kinase C isoform, protein kinase C\(_\gamma\). Representative blots were taken from 3 independent experiments. (C) Downregulation by PMA did not prevent PDGF-induced ERK1/2 activation in the neonatal portal vein. In the fully developed portal vein, PMA downregulation significantly decreased PDGF-induced ERK1/2 phosphorylation (\(n=3\)). Open bars represent neonatal portal vein and filled bars represent fully developed portal vein. Asterisk denotes statistical significance, \(P<0.05\).
mol/L phorbol 12-myristate 13-acetate (PMA) for 48 h to downregulate phorbol ester-sensitive (conventional and novel) protein kinase C isoforms (Fig. 4B). This prolonged incubation did not affect expression of protein kinase Cζ, an atypical protein kinase C isoform. Following PMA incubation, portal vein were stimulated with PDGF for 15 min. In neonatal portal vein, down regulation of the phorbol ester-sensitive protein kinase C isoforms had no effect on PDGF-induced ERK1/2 activation (Fig. 4C). However, in fully differentiated portal vein, 48 h PMA preincubation significantly inhibited PDGF-stimulated ERK1/2 phosphorylation (Fig. 4C) by approximately 70%. This suggests that in neonatal (but not fully differentiated) portal vein, the PDGF-induced ERK1/2 phosphorylation occurs predominantly via an atypical protein kinase C isoform, probably protein kinase Cζ.

3.5. PLCγ expression in balloon-injured rabbit artery

Thin sections of fixed and frozen subclavian arteries were prepared from balloon-injured rabbits. Sections were incubated with anti-PLCγ1 antibody or anti-smooth muscle actin antibody. Tissue sections stained with the anti-PLCγ1 antibody revealed strong cytoplasmic staining in the media of subclavian arteries but generally did not brightly stain cells in the neointimal region (Fig. 5A). The presence of cells in the neointima in these sections was confirmed by BOBO-3 staining of the nuclei. Control subclavian arteries (uninjured) showed an identical pattern of staining in the media compared to medial regions of injured arteries (not shown). In all injured arteries the neointima could be clearly visualized and distinguished from the media by its morphology. Sections stained with smooth muscle-actin antibodies demonstrated that the majority of cells in the neointima were of smooth muscle origin (Fig. 5B).

4. Discussion

It is now evident that changes in both [Ca2+]i and MAP kinase activation (particularly ERK1/2) play an important role in regulating gene expression in VSM [7,10]. A variety of stimuli can induce this growth signal, including growth factors [7], other mitogenic factors and stretch [21]. Exactly how these signals are regulated during phenotypic modulation is not clear. In this study we have examined PDGF-induced regulatory mechanisms in native VSM of different phenotypes. The ability to use freshly isolated native VSM is important as it allows the assessment of phenotype-dependent differences in physiologically relevant models. The rat portal vein provides a more physiologically relevant system to study the mechanisms of phenotypic modulation compared to a cultured cell system, where a fully differentiated VSM phenotype is not possible. In the neonatal portal vein (rat and human) smooth muscle cells are in an undifferentiated, proliferating (hyperplastic) state and resemble myoblasts [22]. At 2–4 days the neonatal portal vein is contractile although has lower levels of smooth muscle-specific proteins [15] and expresses known markers of proliferation [19] as previously demonstrated by our group. Also large nuclei and extensive endoplasmic...
Ca$^{2+}$ homeostasis occurs via activation of PLC. Intracellular mechanism resulting in PDGF-induced signaling pathways involved in regulating gene expression. It has been previously shown that activation of the transcription factor NFATc is dependent on a rise in Ca$^{2+}$, and can be activated by PDGF in VSM [11,12]. The intracellular mechanism resulting in PDGF-induced increases in Ca$^{2+}$ occurs via activation of PLCg leading to InsP3 production [24]. The failure of PDGF to activate NFATc in neonatal portal vein is therefore due, at least in part, to the absence of a PDGF-induced increase in Ca$^{2+}$. This lack of Ca$^{2+}$ increase is probably due to the very low expression of PLCg in proliferating portal vein. This also does not preclude potential changes in expression of intracellular Ca$^{2+}$ channels and Ca$^{2+}$ pumps which have also been shown to occur during phenotypic modulation [15,23]. Our findings are in contrast to a recent study [25] which has demonstrated the importance of PLCg in PDGF signaling in serum-starved primary cultured VSM cells. This may reflect different regulation in cultured versus freshly isolated tissue. Interestingly, PLCg expression is significantly lower in neointimal VSM of balloon-injured rabbits. These neointimal cells are a de-differentiated VSM with a proliferative signal [11], although low expression of PLCg has a general role in determining phenotypic modulation in many cell types, including VSM cells [28]. The results of these studies, taken together with those of the present study, lead to the possibility that PLCg has a potential role in maintaining a differentiated phenotype in native VSM although gain/loss of function experiments will be required to determine if this is the case.

As discussed above, the differences in Ca$^{2+}$ homeostasis has downstream consequences for associated signaling pathways, such as the activation of the Ca$^{2+}$-dependent transcription factor NFATc. Typically, NFATc exists in a phosphorylated state and is activated by dephosphorylation via calcineurin, a Ca$^{2+}$–calmodulin-dependent protein phosphatase [29]. This dephosphorylation allows NFATc to localise to the nucleus and initiate transcription by DNA-binding. In VSM, NFATc is activated both by Ca$^{2+}$ influx and by intracellular Ca$^{2+}$ release induced by agonist stimulation. The relevance of differential activation of NFATc by PDGF observed in this study in proliferating, compared to fully differentiated, VSM is unknown. NFATc-stimulated transcription has been suggested to be associated with a proliferative signal [11], although low expression of PLCg does not preclude NFATc activation by other stimuli, such as 7-transmembrane receptor agonists acting via PLCg (expressed equally in neonate and fully developed portal vein, data not shown). The role of NFATc in neointimal formation has not yet been determined.

The involvement of ERK1/2 in cell proliferation is well described. In cultured smooth muscle cells (both vascular and non-vascular), ERK1/2 is essential for proliferation [30,31]. Regulation of ERK1/2 is critical for determining gene expression [7]. ERK1/2 regulation by PDGF in VSM proliferating, compared to differentiated, phenotypes is therefore of importance to understanding phenotypic modulation in blood vessels. The phosphorylation of ERK1/2 was not affected by differences in the PDGF-induced increase in Ca$^{2+}$, although the activating pathways were altered. In fully differentiated portal vein, PDGF-induced ERK1/2 activation is dependent on an increase in Ca$^{2+}$, and activation of conventional protein kinase C isomers. In actively proliferating VSM (neonatal portal vein), stimulation of ERK1/2 by PDGF is Ca$^{2+}$-independent and likely to be via the atypical protein kinase C isofrom, protein kinase Cζ. In contrast, in cultured VSM cells, activation of ERK1/2 by protein kinase C and Ca$^{2+}$ has been implicated as critical in VSM cell proliferation [31] and may reflect yet more differences between cultured and freshly isolated VSM. Therefore, in the present study despite differences in upstream regulation (as a result of differential Ca$^{2+}$ homeostasis), PDGF-induced ERK1/2 activation is unlikely to directly produce differences in gene expression.
activated via compensatory pathways despite no increase in \([\text{Ca}^{2+}]_i\). This regulation could represent an important regulatory switch involved in the process of phenotypic modulation of VSM.

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**References**


