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

Colin G. Egan\(^a\), Cherry L. Wainwright\(^b\), Roger M. Wadsworth\(^c\), Graeme F. Nixon \(^a\)*

\(^a\)School of Medical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK
\(^b\)School of Pharmacy, Robert Gordon University, Aberdeen, UK
\(^c\)Department of Physiology and Pharmacology, University of Strathclyde, Glasgow, UK

Received 11 January 2005; received in revised form 9 March 2005; accepted 24 March 2005
Available online 10 May 2005

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 (NFAT\(^c\)), 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 NFAT\(^c\). PDGF did not induce NFAT\(^c\) 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.

© 2005 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.

Keywords: Calcium (cellular); Smooth muscle; MAP kinase; Signal transduction

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 μmol/L Fura-2 AM for 40 min in Krebs buffer (in mmol/L: 130 NaCl, 5.6 KCl, 1.2 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 Metafluo 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'-ACGCCCAAGGAAAAATTTGTTC-CATA) 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 artery 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-PLCy1 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+]i and expression of PLCγ

In portal vein myocytes from 6-week-old rats, application of PDGF elicited an increase in the [Ca2+]i. 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+]i, in myocytes from 2- to 4-day-old portal vein (Fig. 1A). However, stimulation with endothelin-1 produced a significant rise in the [Ca2+]i, (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 is

![Graph showing fluorescence ratio (FR) of PDGF on portal vein myocytes.](Image)

**Fig. 1.** (A) Representative fluorescence ratio (FR\(_{340/380}\)) traces of PDGF on [Ca\(^{2+}\)], in fura 2-loaded portal vein myocytes. In 2–4-day-old myocytes, PDGF failed to produce any change in [Ca\(^{2+}\)]\(_i\) (\(n=25\) cells from at least 12 rats) whereas in 6-week-old portal vein, PDGF produced robust Ca\(^{2+}\) increases (\(n=32\) cells from at least 12 rats). In tissue at the same developmental stage as assessed for Ca\(^{2+}\) imaging, PLC\(_{y1}\), PLC\(_{y2}\) and PDGF\(\gamma\) receptor expression were determined. Both PLC\(_{y1}\) and PLC\(_{y2}\) were virtually undetectable in the neonatal portal vein whereas these isoforms were expressed in the fully developed portal veins. PDGF\(\gamma\) receptor expression was unchanged. Representative blots were taken from 4 independent experiments. (B) Representative immunoblots of portal vein whole cell homogenates from 2-, 7-, 14-, 28- and 42-day-old rats stained with antibodies against PLC\(_{y1}\), PCNA and calponin (40 \(\mu\)g/lane, \(n=3\)). PLC\(_{y}\) expression was almost undetectable in 2-day-old portal vein and increased with age reaching a maximum between 28 and 42 days. Calponin expression increased throughout the developmental period examined. PCNA (a marker of proliferating cells\(^{19}\)) was maximally expressed at 2-day-old and decreased with age. Representative blots were taken from 4 independent experiments.

**Fig. 2.** (A) Cytosolic (40 \(\mu\)g protein) and nuclear fractions (20 \(\mu\)g protein) of portal vein homogenates from 2–4-day-old and 6-week-old rats were subjected to immunoblotting with anti-NFATc3 antibody. Following stimulation with PDGF (50 ng/mL) for 30 min, there was a significant decrease in the cytosolic levels of NFATc3 (3.2 ± 0.3 fold, \(n=4\)), and an increase in NFATc3 nuclear expression (2.0 ± 0.2 fold, \(n=4\)) in the 6-week-old portal vein. However, in the neonatal PV, stimulation with PDGF produced no change in cytosolic/nuclear NFATc3 expression. To ensure equal protein loading, cAMP response element-binding protein (CREB) expression was measured in nuclear fractions. CREB is localized to the nucleus in portal vein\(^{19}\) and expression did not change during PDGF stimulation. CREB expression was equal in all samples (\(n=4\)). Equal protein loading was also confirmed by actin staining (not shown). Representative blots were taken from 4 independent experiments. (B) Portal veins from 2–4-day-old rats were stimulated with thapsigargin (1 \(\mu\)mol/L) for 15 min which produces a rise in [Ca\(^{2+}\)]\(_i\) (confirmed by fluorescence imaging). Nuclear fractions (20 \(\mu\)g protein) prepared from homogenates were subjected to immunoblotting with anti-NFATc3 antibody. There was a significant increase in NFATc3 nuclear expression (4.2 ± 2 fold, \(n=4\)) and a significant decrease in the cytosolic levels of NFATc3 (2.2 ± 0.3 fold, \(n=3\)). (C) Nuclear fractions of portal vein (10 \(\mu\)g protein) were subjected to EMSA. In 6-week-old portal vein, PDGF (50 ng/mL) stimulation for 30 min produced an increase in NFATc–DNA binding, compared to control. This was decreased to unstimulated levels by the inclusion of unlabelled probe in the reaction mix. In neonatal portal vein, PDGF stimulation did not increase NFATc–DNA binding. In each gel a positive control nuclear preparation was included (supplied by manufacturer, Panomics, USA) and produced an increase in NFATc–DNA binding. Representative blots were taken from 3 independent experiments.
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). 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 \(\text{Ca}^{2+}\)

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

![Figure 3](https://academic.oup.com/cardiovascres/article-abstract/67/2/308/284775)

Fig. 3. (A) The phospholipase C inhibitor, U73122, significantly inhibited PDGF-induced ERK1/2 phosphorylation in fully developed portal vein but not in neonatal portal vein. Open bars represent 2–4-day-old portal vein and filled bars represent 6-week-old portal vein (n = 3–5 per group). (B) The dependence of PDGF-induced ERK1/2 activation on \([\text{Ca}^{2+}]_i\), in neonatal compared to fully developed portal vein was examined. Pre-incubation of 6-week-old portal vein with BAPTA/AM (30 \(\mu\)mol/L) or 2-APB (10 \(\mu\)mol/L) significantly inhibited PDGF-induced ERK1/2 activation (n = 3–5 per group). In the neonatal portal vein, PDGF-induced ERK1/2 activation was unaffected by pre-incubation with either BAPTA/AM or 2-APB (n = 3–5 per group). Asterisk denotes statistical significance, \(P < 0.05\).
(an intracellular Ca\textsuperscript{2+} chelator) or 2-APB (a partially selective inhibitor of InsP\textsubscript{3}-induced Ca\textsuperscript{2+} release). 2-APB not only blocks InsP\textsubscript{3} receptors and subsequent Ca\textsuperscript{2+} release from stores but also blocks store-operated Ca\textsuperscript{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\textsuperscript{2+}-dependent (conventional) protein kinase C isoforms, Gö 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\textsuperscript{2+}-independent protein kinase C isoform. In order to further determine the protein kinase C isoforms involved, portal vein were treated with 20

---

**Fig. 4.** The role of protein kinase C in ERK1/2 activation was examined. (A) Pre-incubation with Ro-31-8220 (20 μmol/L), a general protein kinase C inhibitor or Gö 69776 (10 μmol/L), an inhibitor of Ca\textsuperscript{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 Gö 69776 (n = 3). (B) Immunoblots showing protein kinase C expression following downregulation of phorbol ester-sensitive protein kinase C (48 h with 20 μmol/L PMA). This prolonged incubation did not affect expression of the atypical protein kinase C isoform, protein kinase Cγ. 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 $[\text{Ca}^{2+}]_\text{i}$ 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

Fig. 5. Expression of PLCγ1 in neointima of injured rabbit subclavian arteries. (A) Confocal photomicrographs of subclavian artery sections (longitudinal) co-stained with anti-PLCγ1 (green) and the nuclear dye BOBO-3 (red). Neointima was determined by examination of transmission image and lack of clearly defined connective tissue layers. In the medial region smooth muscle cells are clearly labeled with the PLCγ1 antibody. However, in the area of neointimal growth very little labeling, compared to normal medium, is observed. Scale bar denotes 5 μm. Typical section of $n=3$ arteries from different rabbits. (B) Confocal photomicrographs of subclavian artery sections (longitudinal) co-stained with anti-smooth muscle (green) and the nuclear dye BOBO-3 (red). Cells in the neointima are positively stained for smooth muscle actin, denoting that the majority of these cells are likely to be of smooth muscle origin. Scale bar denotes 5 μm. Typical section of $n=3$ arteries from different rabbits.
reticulum/golgi apparatus are visible in electron micrographs. This is not the case for arteries, which have fully differentiated (non-hyperplastic) VSM cells at birth although these continue to undergo hypertrophy during postnatal development. Although the portal vein is not a blood vessel with direct relevance to cardiovascular disease, the postnatal developmental properties provide a good model of proliferating VSM [15,19].

The present study has demonstrated that the PDGF-induced increase in \([\text{Ca}^{2+}]\), only occurs in the fully differentiated, but not in the proliferating, portal vein. This difference in the \([\text{Ca}^{2+}]\) homeostasis directly affects downstream 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 \([\text{Ca}^{2+}]\), and can be activated by PDGF in VSM [11,12]. The intracellular mechanism resulting in PDGF-induced increases in \([\text{Ca}^{2+}]\), occurs via activation of PLCγ 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 \([\text{Ca}^{2+}]\). This lack of \([\text{Ca}^{2+}]\) increase is probably due to the very low expression of PLCγ in proliferating portal vein. This also does not preclude potential changes in expression of intracellular \([\text{Ca}^{2+}]\) channels and \([\text{Ca}^{2+}]\) 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 PLCγ in PDGF signaling in serum-starved primary cultured VSM cells. This may reflect different regulation in cultured versus freshly isolated tissue. Interestingly, PLCγ expression is significantly lower in neointimal VSM of balloon-injured rabbits. These neointimal cells are a de-differentiated VSM and will have some similar characteristics to neonatal portal vein. Although further study will be required to determine if PLCγ has a general role in determining phenotypic modulation in VSM, its differential expression could contribute, via differences in \([\text{Ca}^{2+}]\) homeostasis, to a dynamic pattern of gene expression dependent upon growth state. While it is still not clear if this is a primary mechanism for phenotypic regulation, it is likely that the differences in PDGF induced changes in \([\text{Ca}^{2+}]\), will have a role in differentially regulating \([\text{Ca}^{2+}]\)-dependent transcription factors and the associated activation pathways.

The role of PLCγ in VSM growth is not clear. Several studies have demonstrated the important role of PLCγ in regulating differentiation in many cell types. For example, PLCγ1 is required to maintain a differentiated phenotype in keratinocytes and a decrease in PLCγ expression leads to a subsequent decrease in differentiation markers [26]. In addition, overexpression of PLCγ1 in PC12 cells leads to an inhibition of c-fos [27], associated with cell proliferation 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 PLCγ 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 \([\text{Ca}^{2+}]\) homeostasis has downstream consequences for associated signaling pathways, such as the activation of the \([\text{Ca}^{2+}]\)-dependent transcription factor NFATc. Typically, NFATc exists in a phosphorylated state and is activated by dephosphorylation via calcineurin, a \([\text{Ca}^{2+}]\)-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 \([\text{Ca}^{2+}]\) influx and by intracellular \([\text{Ca}^{2+}]\) 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 PLCγ does not preclude NFATc activation by other stimuli, such as 7-transmembrane receptor agonists acting via PLCβ (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 \([\text{Ca}^{2+}]\), although the activating pathways were altered. In fully differentiated portal vein, PDGF-induced ERK1/2 activation is dependent on an increase in \([\text{Ca}^{2+}]\), and activation of conventional protein kinase C isozymes. In actively proliferating VSM (neonatal portal vein), stimulation of ERK1/2 by PDGF is \([\text{Ca}^{2+}]\)-independent and likely to be via the atypical protein kinase C isoform, protein kinase Cζ. In contrast, in cultured VSM cells, activation of ERK1/2 by protein kinase C and \([\text{Ca}^{2+}]\) 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 \([\text{Ca}^{2+}]\) homeostasis), PDGF-induced ERK1/2 activation is unlikely to directly produce differences in gene expression.

In conclusion, PDGF-induced increase in \([\text{Ca}^{2+}]\), observed in fully differentiated VSM does not occur in proliferating VSM. This is, in part, due to low PLCγ expression, which correlates with proliferating VSM phenotypes observed in neonatal developing VSM and neointimal VSM following injury. This differential \([\text{Ca}^{2+}]\) homeostasis has downstream functional effects on the \([\text{Ca}^{2+}]\)-dependent transcription factor NFATc, where no rise in \([\text{Ca}^{2+}]\), prevents PDGF-induced NFATc activation. Other PDGF-induced signaling cascades, such as ERK1/2, are still
activated via compensatory pathways despite no increase in [Ca2+]i. This regulation could represent an important regulatory switch involved in the process of phenotypic modulation of VSM.

Acknowledgement

This study was supported by The British Heart Foundation.

References