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Linking phospholipase C isoforms with differentiation function in human vascular smooth muscle cells☆

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ABSTRACT

The phosphoinositol-phospholipase C (PLC) family of enzymes consists of a number of isoforms, each of which has different cellular functions. PLCγ_1 is primarily linked to tyrosine kinase transduction pathways, whereas PLCδ_1 has been associated with a number of regulatory proteins, including those controlling the cell cycle. Recent studies have shown a central role of PLC in cell organisation and in regulating a wide array of cellular responses. It is of importance to define the precise role of each isoform, and how this changes the functional outcome of the cell. Here we investigated differences in PLC isoform levels and activity in relation to differentiation of human and rat vascular smooth muscle cells. Using Western blotting and PLC activity assay, we show that PLCδ_1 and PLCγ_1 are the predominant isoforms in randomly cycling human vascular smooth muscle cells (HVSMCs). Growth arrest of HVSMCs for seven days of serum deprivation was consistently associated with increases in PLCδ_1 and SM α-actin, whereas there were no changes in PLCγ_1 immuno-reactivity. Organ culture of rat mesenteric arteries in serum free media (SFM), a model of de-differentiation, led to a loss of contractility as well as a loss of contractile proteins (SM α-actin and calponin) and PLCδ_1, and no change in PLCγ_1 immuno-reactivity. Taken together, these data indicate that PLCδ_1 is the predominant PLC isoform in vascular smooth muscle, and confirm that PLCδ_1 expression is affected by conditions that affect the cell cycle, differentiation status and contractile function.

1. Introduction

The phosphoinositol-phospholipase C (PLC) family of enzymes plays an important role in trans-membrane signalling in response to many hormones and growth factors. The primary function of PLC is to catalyse the hydrolysis of phosphatidylinositol 4,5-bisphophate (PIP_2) to generate inositol (1,4,5)triphosphate (IP_3) and 1,2-diacylglycerol (DAG). IP_3 initiates an increase in intracellular calcium, whereas DAG activates protein kinase C (PKC). Control over this important second messenger pathway is key to changes in cellular activity and function.

The PLC family consists of a number of isoforms which regulate different cellular functions. PLCγ_1 activity can be stimulated following growth factor-binding to receptor-linked tyrosine kinases [1] and has a well-established role in mitogenesis [2] and cell migration [3]. A recent study indicates that PLCγ_1 also has a key role in the control in hypoxic Ca^{2+} regulation and contraction in mouse resistance pulmonary arteries (and not mesenteric arteries) [4]. On the other hand, PLCδ_1 has a role in norepinephrine-induced vascular smooth muscle contraction [5], has been implicated in hypertension [6], and is down-regulated in gastric cancers [7]. PLCδ_1 accumulates in the cell nucleus during the G_1/S boundary and in G_0 phases in the cell cycle [8,9], following Ca^{2+} influx following ionomycin treatment [10], and accumulates at the cleavage furrow during cytokinesis [11]. Regulation of PLCδ_1 is complex, and various proteins, including transglutaminase [12], p122rhoA [13], and Ral and calmodulin [14] have been reported to influence its activity. Recent studies have indicated that PLCδ_1 has a role in cytoskeletal organisation as well as cell cycling, although a full understanding of its role is lacking.

Differentiated vascular smooth muscle cells express a characteristic profile of proteins which are essential for contractile function. Following cell culture however, these cells dedifferentiate, lose their contractile ability and exhibit altered expression of contractile proteins such as smooth muscle (SM) α-actin and calponin. PLCδ_1 and PLCγ_1 have been identified in smooth muscle, although their association with smooth muscle cell differentiation has not been investigated. The objective of this study was to investigate the profile of PLC isoforms in human

Abbreviations: PLC, phosphoinositol-phospholipase C; HVSMCs, human vascular smooth muscle cells; SFM, serum free media; RC, randomly cycling

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vascular smooth muscle cells (HVSMCs) and whether there are any associated changes in PLC isoforms following an increase in differentiation by incubation in serum free conditions. This is compared to changes in PLC isoform profile in a second model where cultured rat mesenteric arteries lose their ability to contract and the associated changes of PLC isoforms are measured.

2. Materials & methods

2.1. Collection of human and rat tissues

Anonymised discarded tissue samples of saphenous vein were obtained following surgery and the study was approved by St. Mary's Hospital, Imperial College local research ethics committee and conducted according to the principles expressed in the Declaration of Helsinki. Tissues were obtained in 1998 and 1999. Permission to use the tissues was verbally given, which at the time was approved by St. Mary's, Imperial College ethical committee.

The care and use of all rats in this study was carried out in accordance with UK Home Office regulations, UK Animals (Scientific Procedures) Act of 1986, and the ethical review committee of Imperial College London approved this study.

2.2. Vascular smooth muscle cell culture

HVSMCs were cultured in DMEM buffered with 25 mM HEPES and supplemented with 15% (vol./vol.) foetal calf serum (FCS), 4 mM L-alanyl-L-glutamine (Glutamax-I), 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 μg/ml gentamicin using an explant technique as previously described [15] and characterised as HVSMC on the basis of immuno-cytochemistry. Cell cultures were maintained in a humidified atmosphere of 5% CO_2 (vol./vol.) in air at 37 °C. HVSMCs were routinely used at third passage at a density of 10^6 cells per 75 cm^2 flask unless otherwise stated.

2.3. Modulation of phenotypic status

Passage two HVSMCs were plated onto either 75 cm^2 flask (10^6 cells) or sterile cover slips (2×10^4 cells/slip) and allowed to attach for 24 h in DMEM supplemented with 15% (vol./vol.) FCS. Cells maintained in DMEM supplemented with 15% FCS were routinely used as randomly cycling, control cells. Cells were washed twice with phosphate buffered saline (PBS) prior to incubation in serum-free NCTC containing 25 mM HEPES, 4 mM Glutamax-I, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml gentamicin and 25 mg/ml bovine serum albumin, which is referred to as serum free media (SFM).

2.4. Preparation of cell lysates

HVSMCs were washed twice with ice-cold PBS and scraped into 1 ml of ice-cold lysis buffer (50 mM Tris (pH 7.4); 150 mM NaCl; 1 mM EGTA; 1% (vol./vol.) NP-40; 0.25% sodium deoxycholate; 1 mM sodium fluoride, sodium orthovanadate, phenylmethylsulphonyl fluoride; 1 μg/ml aprotinin, pepstatin, leupeptin) [16,17]. This lysate was vortexed and allowed to stand on ice for 10 min prior to centrifugation (14,000 rpm, 15 min, 4 °C). The protein concentration of the resulting supernatants was determined using the Bradford method.

2.5. Immunoprecipitation

HVSMC cellular protein (200 μg) was removed from all samples and pre-cleared for 1 h with 50 μg albumin-agarose, prior to incubation with antibody (1 μg antibody/100 μg protein) for 3 h at 4 °C. Immunoprecipitates were captured on protein A-agarose as previously described [16].

2.6. Immunoblotting

Proteins were separated on 10% SDS-polyacrylamide gels prior to transfer to nitrocellulose using a Bio-Rad wet gel transfer system. Following successful transfer nitrocellulose blots were blocked in 5% BSA for 1 h and washed three times in Tris buffered saline containing 0.05% Tween 20 (TTBS), prior to probing with primary antibody for 1 h. Blots were washed well in TTBS before being probed with the appropriate secondary antibody and developed using enhanced chemiluminescence.

2.7. DNA synthesis assays

DNA synthesis was determined by measuring the incorporation of [methyl ^{3}H]-thymidine into acid insoluble material [18]. Cells were either maintained in serum-supplemented DMEM or placed in serum-free NCTC for 1, 3, 5 and 7 days prior to assay; [methyl ^{3}H]-thymidine at 1 μCi per well (5 μCi/ml) was added to each well for a six hour period and the experiment was terminated with 10% (wt./vol.) trichloroacetic acid.

2.8. Semi-quantitative PCR

cDNA was prepared from DNase-treated total RNA using the Omniscript RT kit that was amplified using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers by PCR and this was used to normalise cDNA for PCR with other primers. Primers used were: GAPDH sense (5′-TCCCCACTGCCAACGTGTCAG-3′), antisense (5′-GTTGTCATACCAGGAAATGAGC-3′); PLCδ sense (5′-TGCCAGATCGTGGCCCTGAATTTCCAGACAC-3′), antisense (5′-CCTTGCTTGAGGCTGTTCAAGGGGATGGTAC-3′); PLCγ sense (5′-GGAGCTGAGTATGACAGCACCAAGC-3′), antisense (5′-AGAGGCGGTTGTCTCCATTGACC-3′).

2.9. Immunofluorescence

HVSMCs grown on sterile cover slips were washed twice with PBS prior to fixing in 100% methanol for 10 min before being allowed to air dry. Cover slips were then blocked in PBS containing 0.1% of BSA (bovine serum albumin) and 0.1% Triton X-100 (TPBS) for 1 h before the addition of primary antibody (1:50 in blocking solution) for 1 h at room temperature (SM α-actin, PLCγ_1) or overnight at 4 °C (PLCδ_1). These were then washed three times in TPBS before the addition of the appropriate secondary antibody. Cover slips were then washed, mounted onto glass slides and viewed using a Leica TCS SP confocal microscope. Due to the relatively flat nature of these HVSMCs in culture all images shown are maximum projection.

2.10. Measurement of PLC activity

PLC activity was measured using the method previously described [13,19]. Briefly 30 μl aliquots of protein samples were assayed for PLC activity in a final volume of 100 μl containing PLC buffer (20 mM Tris, pH 7.0, 100 mM NaCl, 2 mM EGTA, 2.4 mM $MgCl_2$, 8 mM $CaCl_2$ and 0.05% BSA). The assay was initiated by the addition of 10 μl substrate (unilamellar vesicles containing 350 μM PI, 360 μM phosphatidylethanolamine, 0.015 μCi [^{3}H]-PI). Following a 10 min incubation at 37 °C the reaction was terminated by the addition of 0.5 ml ice-cold chloroform:methanol:HCl (10:10:0.06) followed by 0.15 ml 1 M HCl containing 5 mM EGTA. Samples were vortexed and then left on ice for 10 min. Aliquots of the aqueous phase containing water-soluble hydrolysis products were counted in a Liquid Scintillation Analyser.

2.11. Rat mesenteric artery organ culture

Male Wistar rats (250–300 g) were killed by cervical dislocation and the mesentery isolated. Second or third order branches of the superior mesenteric artery (internal diameter ~200–300 μm) were dissected,

mounted on a single stainless steel wire (2 cm long) and transferred to Bijou bottles containing 5 ml SFM. Vessels were incubated at 37 °C under 5% CO_2 in a water-jacketed incubator for up to 4 days.

2.12. Isometric myography

Fresh or cultured mesenteric arteries were mounted as ring segments in an isometric wire myograph [20] in physiological salt solution (PSS; 119 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.17 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.18 mM KH_2PO_4, 0.027 mM EDTA, 5.5 mM glucose) maintained at 37 °C and aerated with 95% oxygen/5% carbon dioxide. All vessels were allowed to equilibrate for 1 h prior to being set at a 'normalised' internal circumference $0.9.L_{100}$ estimated to be 0.9 times the circumference they would maintain if relaxed and exposed to 100 mm Hg transmural pressure. This was calculated for each individual vessel on the basis of passive length-tension characteristics of the artery and the Laplace relationship [20]. All experiments were initiated by repetitively stimulating vessels for approximately 2 min with a high potassium solution (KPSS; 124 mM, 2.5 mM $CaCl_2$, 1.17 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.18 mM KH_2PO_4, 0.027 mM EDTA, 5.5 mM glucose) until reproducible contractions were elicited. After washout and recovery as appropriate, vessels were then stimulated with norepinephrine (NE; 10 μM) and then 5-hydroxytryptamine (5-HT; 10 μM).

2.13. Protein preparation & analysis of rat mesenteric arteries

Preparation of protein samples from whole vessels was performed as described by Lindqvist et al. [21] with minor modifications. Briefly, fresh and cultured vessels were rinsed well in PSS and cut into 2 mm lengths under the dissecting microscope. Equal numbers of vessels were then placed into Eppendorf tubes containing SDS sample buffer (62.5 mM Tris–HCl, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue) and microwaved for 2 min on full power (1100 W), cooling on ice every 15 s. Samples were then boiled for 10 min and cooled on ice prior to protein separation and Western blotting [16]. For purposes of subsequent analysis, protein immuno-reactivity in fresh mesenteric arteries was taken to be 100% and immuno-reactivity in all cultured arteries was calculated with respect to this.

2.14. Confocal imaging of rat mesenteric arteries

Fresh and cultured vessels were mounted as ring segments in the isometric myograph prior to being fixed in 4% paraformaldehyde solution in calcium-free PSS for 15 min at room temperature. Fixed vessels were dissected longitudinally and washed in PBS (phosphate buffered saline) containing 0.1% vol./vol. Triton X-100 (TPBS) at 4 °C overnight prior to incubation with primary antibody for 18 h at 4 °C. Vessels were then washed thoroughly in TPBS prior to incubation with secondary antibody for 4 h at room temperature. Following this vessels were washed 4 times for 15 min in TPBS at room temperature with 20 nM 4′-6-diamidino-2-phenylindole (DAPI) being added in the final wash. Vessels were then mounted flat, with adventitia facing upwards. Images of the upper adventitial layer and the smooth muscle cell layer were taken with Leica confocal microscope, and the images analysed using Leica Application Suite Advance Fluorescence Lite software. Images were taken of the same section, using the confocal lenses to move through the Z plane. Confocal settings remained the same for all images taken. Images from the inner endothelial cell layer were not recorded as these cells are no longer present following organ culture for 4 days.

2.15. Analysis of data

All data are represented as the mean ± SEM of n observations. Statistical comparisons of data were made using Student's *t*-test for single paired comparisons or one-way repeated measures of variance with Bonferroni's post-hoc test using GraphPad InStat 4.0 as appropriate. p values of less than 0.05 were considered statistically significant.

2.16. Material

Cell culture materials and foetal calf serum were obtained from Life Technologies. Bovine serum albumin fraction V was from Boehringer Mannheim. Hybond C nitrocellulose, streptavidin complex and ECL-film were from Amersham. Antibodies to PLCβ_2, β_3, β_4, γ_2 and δ_2 were obtained from Insight Biotechnology Ltd, and all other antibodies were obtained from TCS. Chemiluminescence reagents were obtained from National Diagnostic and Protein-A agarose was from Cambridge Bioscience. All other reagents were obtained from Sigma.

3. Results

3.1. Characterisation of PLC isoform profile in HVSMCs

The profile of PLC isoform immuno-reactivity in saphenous vein-derived HVSMC was investigated using Western blotting techniques. PLC isoforms β_1, β_2, β_3, β_4, γ_1, γ_2, δ_1 and δ_2 were all detected in samples of rat brain but only PLCγ_1 and PLCδ_1 immuno-reactivity were detected in either fresh saphenous vein, or saphenous vein-derived HVSMC lysates (Fig. 1A).

Immuno-precipitation of either PLCδ_1 or PLCγ_1 from randomly cycling cell lysates resulted in a markedly reduced PLC activity (Fig. 1B), and indicated clearly that the majority if not all PLC activity in these cells can be accounted for by these two isoforms. Consequently investigations into the relative changes in immuno-reactivity of PLC isoforms with modulation of differentiation status were limited to PLCγ_1 and PLCδ_1.

In contrast, analysis of mRNA expression using semi-quantitative RT-PCR techniques indicates that PLCδ_1mRNA and PLCγ_1 mRNA are unchanged by 7 days of serum deprivation (Fig. 1C and D).

3.2. Temporal effects of serum deprivation on DNA synthesis and PLC immuno-reactivity

Incorporation of [^{3}H]-thymidine was measured in both randomly cycling and serum deprived HVSMCs. While the level of [^{3}H]-thymidine incorporation was high in randomly cycling cells it dropped significantly 24 h post serum deprivation, and remained low until day 7 (Fig. 2A).

SM α-actin immuno-reactivity of serum deprived HVSMCs was not significantly different to that seen in randomly cycling control cells over the first 5 days of serum deprivation. However at 7 days post-serum deprivation SM α-actin immuno-reactivity was significantly increased above the level seen in control cells (Fig. 2B). Similarly PLCδ_1 immuno-reactivity in serum deprived cells was similar to that seen in control cells over the first five days but was significantly elevated by 7 days post serum deprivation (Fig. 2B). PLCγ_1 immuno-reactivity levels on the other hand clearly began to decline compared to control cells immediately following serum deprivation although this did not become statistically significant until day 7 (Fig. 2B).

The level of immuno-reactivity of the contractile proteins SM α-actin and calponin are generally accepted to be independent markers of differentiation status in HVSMC. Consequently the immuno-reactivity of these proteins was used to examine the effect of agents used to induce phenotypic modulation. The relative immuno-reactivity of PLCγ_1 and PLCδ_1 isoforms was examined in parallel to this and compared to control, randomly cycling cells.

In randomly cycling cells SM α-actin formed an array of diffuse thin actin filaments which although relatively unstructured were nonetheless visible throughout the cytoplasm. Following prolonged (7-day) serum deprivation SM α-actin formed a clearly defined structure with distinct long, thick fibres running the length of the cells. The staining

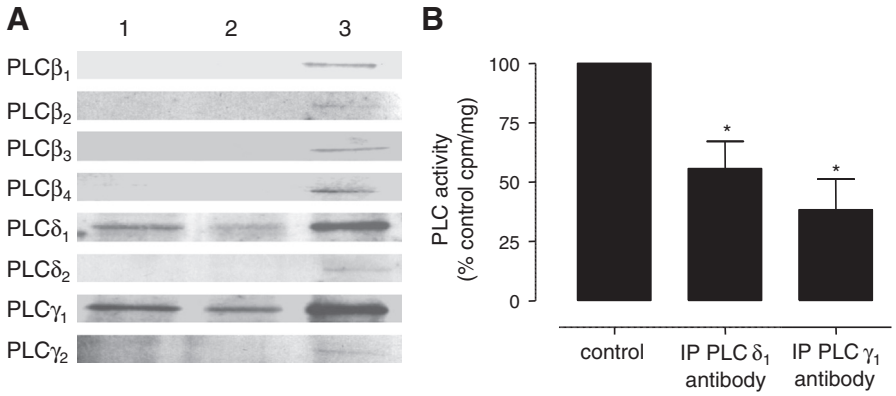


Fig. 1. PLC profile of human vascular smooth muscle cells (HVSMCs). A. Western blot showing the immuno-reactivity of specific PLC isoforms in lysates (30 μg total protein) from 1) fresh and 2) cultured human saphenous vein smooth muscle cells (second passage) and 3) lysate of rat brain. Extracts from either fresh saphenous vein or secondary passaged cells were probed with primary antibody followed by secondary antibody (rabbit anti-mouse-HRP — $PLC\beta_1$, $PLC\delta_1$, $PLC\gamma_1$, swine anti-rabbit-HRP — $PLC\beta_2$, $PLC\beta_3$, $PLC\beta_4$, $PLC\delta_2$, $PLC\gamma_2$). B. PLC activity in lysates of randomly cycling (RC) HVSMC following immuno-precipitation with goat (control), $PLC\delta_1$ and $PLC\gamma_1$ antibodies. PLC activity in lysates treated with goat antibody (control) was taken as 100%. Data is shown as the mean ± SEM (n = 5–6). Significance of $p \leq 0.05$ by one way ANOVA followed by Bonferroni's multiple comparison post hoc test is indicated by * compared to a theoretical median in randomly cycling cells (RC) of 100%.

was less evenly distributed, being stronger in the perinuclear region than at the cell periphery (Fig. 3).

These changes were paralleled by a clear shift in $PLC\delta_1$ localisation from the perinuclear region to the entire cytoplasmic region. $PLC\delta_1$ also appeared to take on a more structured appearance in growth arrested cells, with the formation of some distinct fibres, which might be suggestive of some association with the structural components of the cell (Fig. 3). $PLC\gamma_1$ on the other hand demonstrated a very structured appearance in randomly cycling control cells with fibres appearing to run the length of the cell. In serum deprived cells however $PLC\gamma_1$ binding was dramatically reduced, largely restricted to the perinuclear region and of a more granular appearance (Fig. 3). Dual staining of $PLC\delta_1$ and SM α-actin shows that changes in distribution clearly change with increases in differentiation of the cell, but there is no evidence of co-localisation between these proteins.

3.3. Temporal effects of organ culture on force generation by rat mesenteric artery

The contractile force of all cultured vessels, in response to a high $[K^+]$ (Fig. 4A) solution, NA (Fig. 4B) and 5-HT (Fig. 4C) was maintained for the first 3 days, but was significantly different by day 4 when compared to that of fresh arteries.

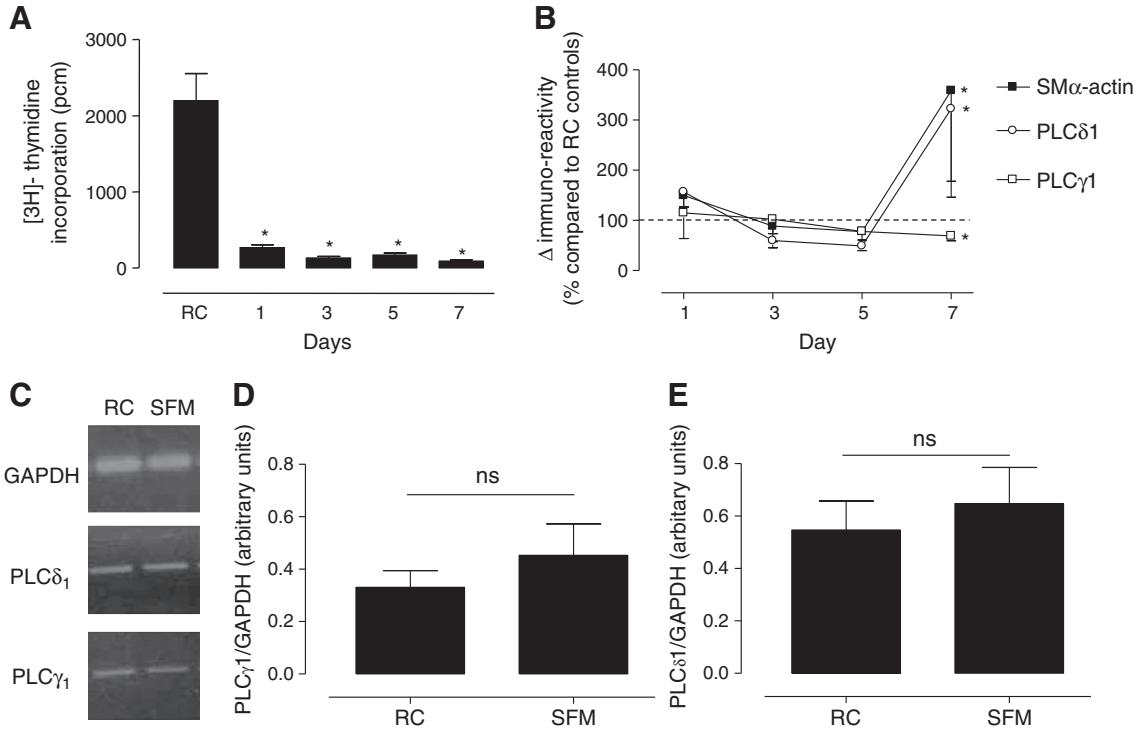


Fig. 2. Temporal changes during growth arrest to DNA replication and expression of proteins A. DNA replication in growth arrested HVSMC. Graph shows $[^3H]$-thymidine incorporation in randomly cycling cells and following serum deprivation for 1, 3, 5 and 7 days compared to randomly cycling (RC) cells (day 0). Data is shown as the mean ± SEM (n = 4–8 separate cell lines; 6 replicates). B. Changes in immuno-reactivity of SM α-actin, $PLC\delta_1$ and $PLC\gamma_1$ over seven days of serum deprivation. Data are represented as mean % ± SEM in immuno-reactivity on days 1, 3, 5 and 7 following serum free media treatment compared to randomly cycling cells. * = $p < 0.05$ by paired Student's *t*-test; n = 4–8 for each group. C. mRNA expression of GAPDH, $PLC\delta_1$ and $PLC\gamma_1$ in randomly cycling (RC) cells, and cell's serum deprived for 7 days (SFM). D. Densitometry analysis of mRNA; mRNA expression data is expressed as the mean ± SEM; ns indicates no significant difference by one way ANOVA, n = 8.

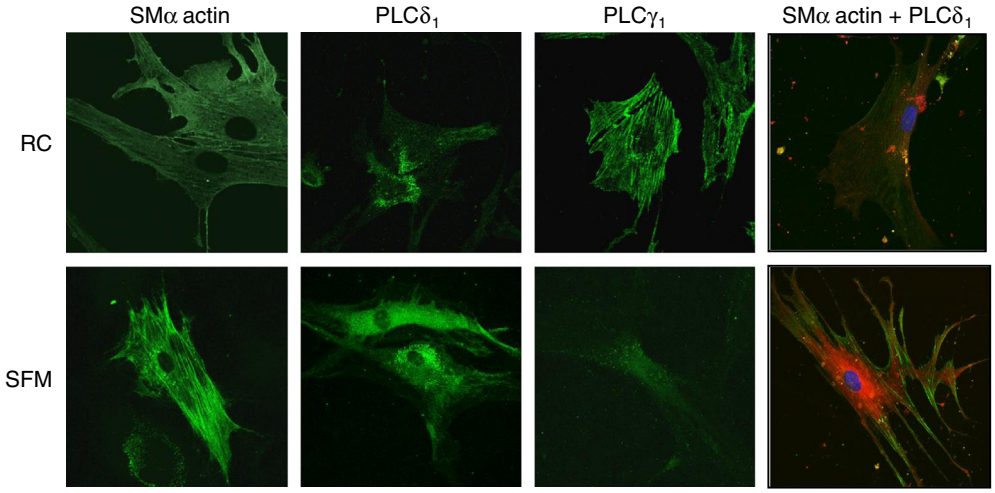


Fig. 3. Effect of prolonged serum deprivation on SM α-actin, PLCδ1 and PLCγ1 protein immuno-reactivity and organisation. Confocal immuno-fluorescence microscopy depicting the sub-cellular localisation and structural organisation of SM α-actin, $PLC\delta_1$ and $PLC\gamma_1$; double staining for SM α-actin (green) and $PLC\delta_1$ (red) in randomly cycling (RC) and serum deprived HVSMC (SFM) taken on day 7 of culture. Blue DAPI staining indicates the nuclear staining. Images presented are maximum projection images (×630) obtained from a single experiment and are representative of n = 3–4 separate experiments with cells from different individuals.

3.4. Effect of culture conditions on protein immuno-reactivity in rat mesenteric arteries

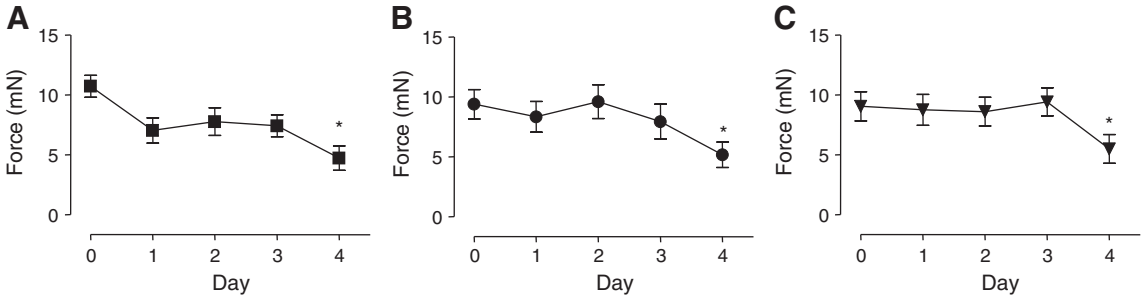
Following 4 days of culture in SFM, rat mesenteric arteries have a significant decrease in SM α-actin, calponin and $PLC\delta_1$ immuno-reactivity compared to fresh arteries (Fig. 4C and D). In contrast, the immuno-reactivity of $PLC\gamma_1$ was not significantly altered in vessels cultured in SFM compared to fresh vessels (Fig. 4C and D).

3.5. Confocal microscopy

Immuno-reactivity to $PLC\delta_1$ was clearly detected in both the adventitial and medial layers of fresh rat mesenteric arteries. Organ culture in SFM for 4 days consistently resulted in a decrease in staining intensity of $PLC\delta_1$ in the smooth muscle cell layer and adventitia (Fig. 5A and B). $PLC\gamma_1$ protein immuno-reactivity was detected in the adventitia and smooth muscle cell layers of fresh vessels. The intensity of $PLC\gamma_1$ staining in smooth muscle cells was not significantly altered in vessels cultured for 4 days in SFM (Fig. 5A and C), and was significantly reduced in the adventitia.

4. Discussion

The PI-PLC family of enzymes is key mediator in the signal transduction of a great array of agonists, and has a central role in cellular function. As a family they are ubiquitous, although it is now clear that cells



	SFM	n=
SMα-actin	82.7 ± 6.2*	11
calponin	52.2 ± 17.8*	10
$PLC\delta_1$	65.2 ± 11.2*	8
$PLC\gamma_1$	114.7 ± 39.5 (ns)	12

Fig. 4. Effects of organ culture on contractility and protein immune-reactivity of rat mesenteric arteries. Arteries were incubated for up to 4 days in SFM and compared to fresh arteries (day 0). Graphs show the maximum contraction in 2 min following the addition of A. KPSS; B. 10 μM noradrenaline (NE); and C. 10 μM 5-hydroxythymidine (5-HT). Data are expressed as mean force (mN) ± SEM of n = 7–9 separate mesenteric arteries; results were compared by one-way ANOVA and * represents a significant difference $p \leq 0.05$ by Bonferonni's post-hoc test of arteries compared to arteries at day 0. D. Smooth muscle α-actin, calponin, $PLC\delta_1$ and $PLC\gamma_1$ immuno-reactivities by Western blotting were detected in extracts from arteries incubated for 4 days in SFM. E. Data are expressed as % of the protein immuno-reactivity levels in freshly isolated mesenteric arteries which was taken to be 100%. Non-significance (ns) and significance of $p \leq 0.05$ by *t*-test are indicated by * compared to a theoretical median of 100%.

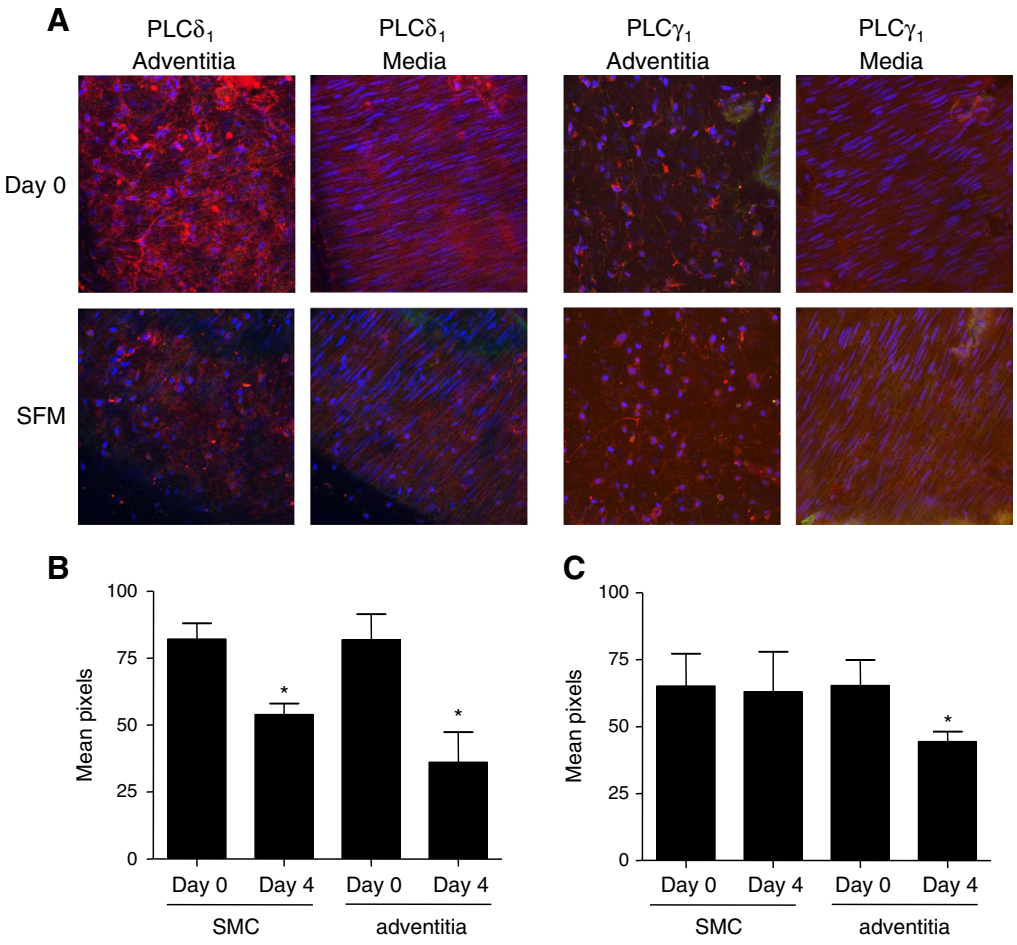


Fig. 5. The effect of organ culture on adventitial and smooth muscle cell immuno-reactivity of PLCδ1 and PLCγ1. A. Confocal images were taken of the medial smooth muscle cell layer or adventitial cells of the same section, using the focus to move through the Z plane. Quantified immuno-reactivity of A. $PLC\delta_1$ and B. $PLC\gamma_1$ in the rat mesenteric artery following 4 day organ culture in SFM. Data is expressed as the mean ± SEM intensity of the staining, shown as mean pixels; significance is indicated * = $p < 0.05$ by unpaired Student's *t*-test comparing to fresh tissue (day 0); n = 3–5.

are isoform specific. Here we present data that indicates that HVSMCs express functional $PLC\delta_1$ and $PLC\gamma_1$ and no other PLC isoform. However, HVSMCs are not representative of the entire cardiovascular system, for example other studies have clearly shown that mouse heart cell lines express predominately PLCβ isoforms [22], whereas HUVECs express all known isoforms except for $PLC\beta_1$, PLCε and PLCξ [23] and that expression of each isoform changes considerably following initiation of inflammatory response to LPS challenge [24].

Of all the PLC isoforms looked for in fresh saphenous vein and the derived cultured cells, only immuno-reactivity of $PLC\delta_1$ and that of $PLC\gamma_1$ were detectable, and the activity of these two isoforms accounts for the total activity of cellular PLC. This is in keeping with what has previously been reported, where $PLC\delta_1$ is the predominant isoform in blood vessels and primary cultured rabbit aortic VSMC [25,26], which is markedly up-regulated by growth arrest by long term serum free conditions. In comparison, $PLC\gamma_1$ immuno-reactivity was reduced compared to randomly cycling cells in these studies.

In contrast to the changes in immuno-reactivity and PLC activity, the mRNA expression of $PLC\delta_1$ and $PLC\gamma_1$ remained the same after 7 days of serum deprivation. This is in keeping with previous findings, where $PLC\delta_1$ and $PLC\gamma_1$ mRNA levels did not correlate with protein expression and activity in a rat heart model [27].

The inability to detect PLCβ isoforms in HVSMC is consistent with previous reports by other groups who failed to detect PLCβ in rat, rabbit or bovine aorta [25,26,19,28]. In contrast two studies have demonstrated the presence of isoforms of PLCβ in porcine aorta [29] and immortalised human aortic cells [30].

Perhaps more importantly, temporal analysis of the changes in PLC isoform immuno-reactivity following serum deprivation of HVSMCs indicated differences in the time course of change in $PLC\delta_1$ and $PLC\gamma_1$. An increase in $PLC\delta_1$ immuno-reactivity did not occur until several days post-growth arrest, which exhibited a temporal profile very similar to that of the increase in SM α-actin; whereas changes in $PLC\gamma_1$ immuno-reactivity were evident 24 h following serum removal, which corresponded with the most marked reduction in thymidine uptake. Furthermore, the sub-cellular localisation of these PLC isoforms differed in randomly cycling cells and was differentially affected by prolonged serum deprivation. In randomly cycling cells $PLC\delta_1$ was expressed throughout the cytoplasm; following serum deprivation expression was increased, and took on a more granular appearance in the perinuclear regions of the cells. This is in keeping with the reported changes in nuclear and perinuclear accumulation of $PLC\delta_1$ throughout the cell cycle in fibroblasts [31]. In contrast, $PLC\gamma_1$ immuno-reactivity was located throughout the cytoplasm, in a filamentous manner in randomly cycling cells, and was more evident in the perinuclear region after serum deprivation. Serum deprivation resulted in changes in SM α-actin and calponin organisation similar to those reported previously in differentiated vascular smooth muscle cells in culture [32].

The cultured rat mesenteric artery is a model of loss of contractility and decreasing smooth muscle differentiation. Loss of contractility in rat mesenteric arteries was associated with a reduction of SM α-actinand calponin as anticipated. $PLC\delta_1$ was highly expressed in fresh vascular tissue [25,26] and both Western blotting and confocal studies indicated that there was a marked reduction in $PLC\delta_1$ and no change in $PLC\gamma_1$

associated with organ culture. Loss of contractility was not coupled to any significant increases in the level of apoptosis of smooth muscle cells (data not shown). There was a close temporal relationship between the reduction in PLCδ_1 immuno-reactivity and reduced contractility and it is therefore tempting to suggest that these two phenomena may be mechanistically related, although this hypothesis requires further study.

The data presented here is the first to show the PLC isoform profile of fresh and cultured human saphenous vein smooth muscle cells. Our findings show that changes in PLC$\delta_1$have a functional significance, and suggest that preventing the loss of PLCδ_1 activity from smooth muscle cells may have beneficial effects.

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