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STORE-OPERATED Ca^{2+} ENTRY IS NOT ESSENTIAL FOR PDGF-BB INDUCED PHENOTYPE MODULATION IN RAT AORTIC SMOOTH MUSCLE

Craig A. Emter^{1,2,*} and Douglas K. Bowles^{1,3}

¹Department of Biomedical Science, University of Missouri- Columbia, 1600 E. Rollins, W160 Veterinary Medicine, Columbia, MO 65211

²Department of Molecular Pharmacology and Physiology, University of Missouri- Columbia, 1600 E. Rollins, W160 Veterinary Medicine, Columbia, MO 65211

³Dalton Cardiovascular Research Center, University of Missouri- Columbia, 1600 E. Rollins, W160 Veterinary Medicine, Columbia, MO 65211

SUMMARY

Suppression of smooth muscle cell (SMC) differentiation marker genes is central to SMC phenotype modulation during vasculo-proliferative diseases such as atherosclerosis and restenosis.

Upregulation of the intermediate-conductance Ca^{2+} -activated K^{+} channel ($\text{K}_{\text{Ca}3.1}$) is integral for mitogen induced suppression of SMC marker genes and post-angioplasty restenosis. Modulation of SMC marker gene expression has been observed following Ca^{2+} influx from multiple sources, however, it's unknown whether upregulation of $\text{K}_{\text{Ca}3.1}$ and/or suppression of SMC differentiation genes is dependent on a Ca^{2+} mediated mechanism. The purpose of this study was to determine the dependence of mitogen induced SMC phenotype modulation on store-operated Ca^{2+} entry (SOCE). In growth-arrested, differentiated rat aortic SMCs, platelet-derived growth factor-BB (PDGF-BB) augmented SOCE. However, PDGF-BB induced upregulation of $\text{K}_{\text{Ca}3.1}$ and downregulation of the SMC marker gene smooth muscle myosin heavy chain (SMMHC) and myocardin was not dependent on SOCE. Co-treatment with the iPLA_2 inhibitor bromoenol lactone (BEL) inhibited the effects of PDGF-BB on SMC phenotype modulation and SOCE. Our results indicate SOCE is not required for PDGF-BB induced phenotype modulation in rat aortic SMCs. Rather, we implicate a novel BEL-sensitive mechanism which regulates both SOCE and phenotype modulation, independently.

Keywords

store-operated Ca^{2+} entry; $\text{K}_{\text{Ca}3.1}$; phenotype modulation; smooth muscle

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*Corresponding author: Craig A. Emter, Department of Biomedical Science, University of Missouri-Columbia, 1600 E. Rollins, E117 Veterinary Medicine, Columbia, MO 65211, Phone: 573-882-4138, Fax: 573-884-6890, emterc@missouri.edu .

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INTRODUCTION

Coronary heart disease is estimated to affect nearly 16 million Americans with an estimated cost of treatment in 2008 of \$156.4 billion [1]. From 1979-2005, cardiac catheterizations increased 342% and approximately 1.26 million percutaneous coronary interventions (PCI) were performed in 2005 in the United States alone [1]. The prevalence of coronary artery disease (CAD) has prompted an abundance of research into the mechanisms involved in not only the formation of atherosclerotic plaques, but also into restenosis following PCI. The development and use of stents in interventional cardiology, though revolutionary in the treatment of coronary artery disease, has been complicated by reports of restenosis within the stent [2,3]. Drug eluting stents have proven effective in the elimination of early restenosis, but are still associated with high rates of late restenosis and thrombosis [2,3]. Consequently, the prevention of in-stent restenosis has become a high priority in the treatment of CAD.

Increased proliferation of vascular smooth muscle cells (SMC) and suppression of SMC marker genes are characteristic of the SMC response to vasculo-proliferative diseases such as atherosclerosis and restenosis [4]. Recently, considerable interest in a new paradigm termed excitation-transcription coupling [5] has focused on defining how the plasticity of ion channels influences gene expression in vascular SMC. In contrast to other cells, SMCs are not terminally differentiated and can alter their genetic profile during vascular development, remodeling, and disease in a process called phenotype modulation [4]. Our laboratory has recently demonstrated upregulation of the intermediate-conductance Ca^{2+} -activated K^+ channel ($\text{K}_{\text{Ca}3.1}$) as integral for mitogen induced suppression of SMC marker genes, including smooth muscle myosin heavy chain (SMMHC), smooth muscle alpha actin ($\text{SM}\alpha\text{A}$), and smoothelin [6]. Further, pharmaceutical blockade of this channel limited stenosis and SMC phenotype modulation in porcine and rat models of angioplasty-induced vascular injury [7,8] and was beneficial in treating atherosclerosis in $\text{ApoE}^{-/-}$ mice [9]. Transcriptional regulation of $\text{K}_{\text{Ca}3.1}$ expression occurs, in part, through AP-1, a transcriptional complex stimulated by increases in intracellular Ca^{2+} [10]. Proliferation of vascular SMCs is Ca^{2+} dependent [11-16], with observed increases in store-operated Ca^{2+} entry (SOCE) in response to growth factors [11,12,17,18] and vascular injury [19] being implicated as key in the modulation of SMC phenotype.

Previous studies have demonstrated modulation in SMC gene expression following Ca^{2+} influx from multiple sources, including L-type [20] and SOC [21-23] channels. Therefore, the purpose of this study was to determine the dependence of mitogen induced modulation of $\text{K}_{\text{Ca}3.1}$, SMMHC, and myocardin on mitogen-augmented SOCE in vascular SMCs. Platelet-derived growth factor-BB (PDGF-BB), a powerful regulator of both SMC marker genes and $\text{K}_{\text{Ca}3.1}$ [6,24], was used to modulate phenotype in rat aortic SMCs in the presence and absence of SOCE blockers. We hypothesized that our previously observed PDGF-BB-induced upregulation of $\text{K}_{\text{Ca}3.1}$ mRNA expression [6] is dependent on Ca^{2+} entry via store-operated mechanisms. On the contrary, our results indicate mitogen-induced modulation of phenotype is not dependent on SOCE. Rather, our findings implicate a novel BEL-sensitive mechanism which independently regulates both SOCE and phenotype modulation.

METHODS

Rat aortic smooth muscle cell (RASMC) culture

Taking advantage of an established and thoroughly characterized model [4,5,25], primary cultures of SMCs were isolated from the medial portion of male Sprague Dawley rat aorta, following removal of the adventitia and endothelium (by mechanical denudation). Animal protocols were approved by the University of Missouri Animal Care and Use Committee. Cells were plated at 1.5×10^4 cells/cm² in DMEM/F-12 media (Invitrogen/GIBCO 11320-033) containing 100 U/ml penicillin/streptomycin, 1.6 mM L-glutamine, and 10% FBS for 3-4 days

until 70-80% confluent, changing media every 2 days. Cells (passages 2-10) were then serum restricted for 4 days to maximize expression levels of smooth muscle differentiation marker genes (SMMHC SM α A, and the transcription factor myocardin) as previous work indicates this process results in maximum expression of these genetic markers allowing accurate assessment of the effects of PDGF-BB on differentiated SMC [25]. Two cell lines and a minimum of 2-3 passages (3 experiments/passage) were used for each treatment. Group comparisons revealed passage number had no effect on experimental results, therefore, data from all experiments were combined.

Ca²⁺Imaging

Coverslip plated RASMCs (70-80% confluent, 4 day serum restricted) were exposed to PDGF-BB (30 ng/mL) or control conditions for 24 or 48 hours. At the time of the experiments, RASMC's were ~100% confluent and myocytes from inside this monolayer were used for analysis. Cells were loaded with FURA2-AM (Molecular Probes) for 30 minutes in a low Ca²⁺ solution (in mM: 0.1 CaCl₂, 125 NaCl, 1 MgCl₂, 5 KCl, 0.44 KH₂PO₄, 0.34 Na₂HPO₄, 2.6 NaHCO₃, 20 HEPES, 5 Glucose, 0.0001% Phenol Red, 1% penicillin/streptomycin, stock amino acids and vitamins-Invitrogen, pH 7.4) and intracellular Ca²⁺ concentrations (F₃₄₀/F₃₈₀ ratio) and rate of entry (Mn²⁺ quench; decline in F₃₆₀) were measured using a dual excitation fluorescence imaging system (Intracellular Imaging, Inc) under 10x magnification. Baseline fluorescence levels were measured for 1 minute, after which cells were exposed to cyclopiazonic acid (CPA; 10 μ M) in Ca²⁺-free solution (in mM: 140 NaCl, 1 MgCl₂, 5 KCl, 10 HEPES, 10⁻⁸ EGTA, 10 glucose, pH 7.4) for 10 minutes to deplete sarcoplasmic reticulum (SR) Ca²⁺ stores. SOCE was elicited by the addition of 2 mM extracellular Ca²⁺ (in mM: 2 CaCl₂, 138 NaCl, 1 MgCl₂, 5 KCl, 10 HEPES, 10 glucose, pH 7.4) or 1 mM Mn²⁺ (added to Ca²⁺-free solution) for 5 minutes. To further confirm Ca²⁺ entry was indeed store-operated, 10 μ M Gd³⁺ [15,26] was applied for 5 minutes following the re-addition of Ca²⁺. All cells on each coverslip were simultaneously exposed to solutions and blockers. Given the molecular diversity of components comprising SOC channels from different vascular beds, we also applied other reported blockers of SOCE. Ni²⁺, 2-APB, and La³⁺, had no effect on SOCE in cultured RASMC's [data not shown; for review see 15]. All solutions contained 10 μ M nifedipine to eliminate Ca²⁺ entry through L-type channels. Changes in intracellular Ca²⁺ and rate of entry were continuously monitored over the course of the protocol. Ca²⁺ concentrations were calculated from an external standard curve generated before the start of daily experiments (F₃₄₀/F₃₈₀ ratio vs. [Ca²⁺] of 0, 38, 100, 225, 351, and 602 nM). Mn²⁺ quench curves (decline in F₃₆₀) were fit with a linear regression trend-line and the slope was used to determine rate of Ca²⁺ entry. Data were excluded from the study if the regression line correlation coefficient was r² < 0.98. To determine the dependence of SOCE on iPLA₂-mediated mechanisms, cells were pretreated with 25 μ M bromoenol lactone (BEL- an irreversible inhibitor of iPLA₂) in low Ca²⁺ solution for 30 minutes at 37°C. BEL was then washed away for 10 minutes in Ca²⁺-free solution before the beginning of the experiment. At least 25 individual RASMCs were analyzed for each experiment.

qRT-PCR

qRT-PCR was performed as previously described (6, 8). Cultured cells in TRIzol solution were quick frozen in liquid nitrogen and stored at -80°C until processed. Total RNA was isolated according to the manufacturer's published protocol for TRIzol. cDNA was transcribed from total RNA using Superscript III RT kit (Invitrogen) in a 20 μ l reaction containing 200U RT, 100 ng of random hexamers, 5 mM MgCl₂, 1 mM dNTPs, and 20 mM DTT. A minus RT reaction was also performed to ensure no genomic DNA contamination. qRT-PCR was performed on a BioRad MyIQ cycler (model no. SC1000-1). Each 25 μ l reaction contained 1 X SYBER Green Master Mix (Qiagen), 0.8 μ M forward and reverse primers, and 1 μ g of cDNA. The reaction conditions were optimized for each set of primers: K_{Ca}3.1, SMMHC,

myocardin, iPLA₂, and 18S (for the primer sequences, refer to Table 1). Gene expression was measured after 4 and 8 hours in untreated control (CNT) and PDGF-BB treated (30 ng/mL) RASMCs simultaneously treated with one of the following conditions: 25 μ M BEL, 10 μ M Gd³⁺, 1.5 mM EGTA, or 25 μ M methyl arachidonyl fluorophosphonate (MAFP). Target gene expression was normalized to 18S ribosomal RNA using the 2^{- $\Delta\Delta$ Ct} method [27]. Linearity and efficiency of each PCR condition were verified by creating a standard curve plotting the critical threshold vs. log of the cDNA dilution.

Statistical analysis

All data analysis was performed using SPSS version 13.0 or SigmaStat version 3.5. All comparisons between groups were made using a 2 (mitogen) $\times$ 2 (pharmacological intervention) ANOVA. Specific group differences revealed by ANOVA were compared by 2-tailed independent or paired samples t-test. All data are presented as means $\pm$ SE. Significance was defined as $P < 0.05$.

RESULTS

PDGF-BB augments store-operated Ca²⁺ entry (SOCE) in RASMCs

Representative traces from control (CNT) and PDGF-BB treated RASMCs illustrating changes in intracellular Ca²⁺ concentrations ([Ca²⁺]_{intra}) are shown in Figure 1A. In the presence of 10 μ M nifedipine, 10 μ M CPA was added for the first 10 minutes, followed by readdition of 2 mM Ca²⁺ and application of 10 μ M Gd³⁺ (a SOCE blocker) as indicated. Summary data from all experiments for peak [Ca²⁺] are presented in Fig 1B. Treatment time (24 or 48 hours) with PDGF-BB showed no significant differences (2 $\times$ 2 ANOVA, treatment time- 24 & 48 HR vs. treatment- CNT & PDGF-BB; treatment time N.S.), thus, these time points were combined. In PDGF-BB treated cells, CPA increased peak [Ca²⁺]_{intra} while no change was observed in CNT cells (Fig. 1B). Any increases in fluorescence during CPA exposure returned to baseline levels before the end of the 10 minute exposure time. After the addition of 2mM extracellular Ca²⁺, [Ca²⁺]_{intra} was elevated in both groups, but to a larger extent in PDGF-BB treated cells (Fig. 1B, Ca²⁺ MAX). Blockage of SOCE with Gd³⁺ significantly reduced [Ca²⁺]_{intra} in both groups (Fig. 1B). PDGF-BB treated cells also showed a greater increase in intracellular Ca²⁺ levels measured as the difference between baseline2 and Ca²⁺ MAX levels (Fig. 1B- bar graph) when compared to CNT cells.

PDGF-BB also increased the maximal rate of CPA-induced SOCE, as demonstrated by representative traces in Figure 2A and summarized data in Figure 2B. Extracellular Mn²⁺ significantly quenched F₃₆₀ fluorescence at a greater rate in PDGF-BB treated cells (Fig. 2B). This effect was seen in 5 of 7 passages (15 of 19 experiments). Experiments in which cells were not exposed to CPA showed no change in the rate of Mn²⁺ influx (Fig. 2B, NO CPA) confirming increases in Mn²⁺ influx rate were the result of emptying of intracellular SR Ca²⁺ stores and subsequent SOCE. Interestingly, the iPLA₂ inhibitor BEL (Fig. 2A & B, BEL) completely inhibited SOCE in both CNT and PDGF-BB treated cells.

RASMC phenotype modulation is BEL sensitive

Previous studies from our laboratory have demonstrated a PDGF-BB-induced increase in K_{Ca}3.1 mRNA expression and decreases in SMMHC and myocardin expression in porcine coronary artery smooth muscle cell culture [6]. Our current results confirmed these findings in RASMCs as treatment with PDGF-BB increased K_{Ca}3.1 mRNA expression significantly after 4 and 8 hours (Fig. 3A). Co-incubation with the irreversible iPLA₂ inhibitor BEL completely blocked this effect at both time points. PDGF-BB also decreased SMMHC (Fig. 3B) and myocardin (Fig. 3C) after 8 hours, consistent with previously established time frames from our laboratory [6]. In contrast, co-treatment with BEL in both the CNT and PDGF-BB

cells increased SMMHC (Fig. 3B) and myocardin (Fig. 3C) mRNA expression at both treatment times and prevented downregulation by PDGF-BB.

Although BEL is a well accepted inhibitor of $iPLA_2$, it is also known to inhibit phosphatidic acid phosphohydrolase-1 (PAP-1) [28-30]. Therefore, RASMCs were also treated with 25 μ M MAFP, a specific $iPLA_2$ inhibitor. MAFP attenuated but did not block $K_{Ca}3.1$ upregulation in cells co-treated with PDGF-BB (Fig. 4A). Myocardin and SMMHC expression was reduced after 8 hours by PDGF-BB and MAFP (Fig. 4B & 4C). In contrast to treatment with BEL, PDGF-BB induced downregulation of SMMHC was not inhibited by MAFP. In fact, the effects of PDGF-BB on myocardin mRNA were enhanced by co-treatment with MAFP. PDGF-BB had no effect on the expression of $iPLA_2$ mRNA (data not shown).

PDGF-BB-augmented SOCE is not required for PDGF-BB-induced RASMC phenotype modulation

In order to determine if mitogen augmented increases in $[Ca^{2+}]_{intra}$ via SOCE were necessary for phenotype modulation, RASMCs were treated with the SOCE blocker Gd^{3+} (Fig. 5) and by removal of $[Ca^{2+}]_{extra}$ using EGTA (Fig. 6) in the presence and absence of PDGF-BB. PDGF-BB increased $K_{Ca}3.1$ mRNA expression and co-incubation with Gd^{3+} enhanced this effect at 8 hours (Fig. 5A). Gd^{3+} alone significantly increased $K_{Ca}3.1$ mRNA expression at 8 hours (Fig. 5A). Treatment with Gd^{3+} also failed to inhibit PDGF-BB induced decreases in SMMHC or myocardin mRNA expression (Fig. 5B & C).

A similar effect was seen when extracellular Ca^{2+} ($[Ca^{2+}]_{extra}$) was chelated by EGTA, reducing effective $[Ca^{2+}]_{extra}$ to approximately 750 nM (as calculated using MaxChelator, Ver. 2.5, <http://www.stanford.edu/~cpatton/>). Chelating $[Ca^{2+}]_{extra}$ with EGTA failed to inhibit either the upregulation of $K_{Ca}3.1$ mRNA (Fig. 6A) or the downregulation of SMMHC and myocardin mRNA (Fig. 6B & C).

DISCUSSION

The results of our study illustrate several novel findings: 1) SOCE is not required for PDGF-BB induced phenotype modulation in RASMCs; 2) both PDGF-BB induced phenotype modulation and SOCE are independently regulated by a BEL-sensitive mechanism; and 3) PDGF-BB augments BEL-sensitive SOCE in growth-arrested, differentiated RASMCs. The importance of vascular smooth muscle cell (SMC) plasticity in the regulation of normal and pathophysiological blood vessel function has been clearly established (for review, see [4]). An abundance of evidence has demonstrated this plasticity enables vascular SMCs to alter the expression of marker genes associated with a differentiated state to gene profiles characteristic of a proliferative, synthetic phenotype [4,5]. This unique change in gene expression is central to the vascular remodeling seen in hypertension, atherosclerosis, and postangioplasty restenosis [4]. Our laboratory has previously implicated changes in plasma membrane ion channel expression as key in the regulation of coronary SMC phenotype. Specifically, upregulation of the intermediate-conductance Ca^{2+} -activated K^+ channel ($K_{Ca}3.1$) appears to be an integral mediator of mitogen-induced SMC de-differentiation [6,8]. L-type voltage-gated Ca^{2+} channels have been implicated in the upregulation of SMC-specific differentiation marker genes in RASMC, enhancing a differentiated SMC phenotype [5,20]. Conversely, increases in store-operated Ca^{2+} entry (SOCE) and the molecular components thought to comprise SOC channels has been observed in proliferating pulmonary artery SMCs [17,18] and injured mouse carotid artery [19]. However, the potential role of mitogen-induced SOCE on suppression of SMC differentiation marker genes, i.e. de-differentiation, has not been reported. We previously hypothesized that growth-factor-induced increases in SOCE and subsequently elevated intracellular Ca^{2+} levels drive expression of $K_{Ca}3.1$ in a positive-feedback manner [6]. Yet, it remained unknown whether the initial upregulation of $K_{Ca}3.1$ expression is dependent on

mitogen-augmented increases in SOCE. Therefore, the purpose of the current study was to determine the role of mitogen-augmented SOCE in the regulation $K_{Ca3.1}$ and SM-specific marker genes representative of a differentiated phenotype in RASMCs.

Vascular SMC dependence on Ca^{2+} in the regulation of proliferation and in the cell cycle is well established [11-16]. Increased intracellular Ca^{2+} in RASMCs [31,32] and activation of a Ca^{2+} permeable, voltage-independent, non-selective cation current in rat mesangial cells [33] by PDGF-BB provides evidence for a Ca^{2+} dependent mechanism by which growth factors induce proliferation. Further, platelet-derived growth factor-BB (PDGF-BB) is a strong modulator of SMC phenotype [34-37], particularly in cell culture, and is increased following vascular injury, including upregulation of PDGF receptor- β (reviewed in [24]). Numerous studies have shown increased SOCE following rat carotid artery injury [19] and emptying of SR Ca^{2+} stores in PDGF-BB treated and proliferating (in serum growth media) rat pulmonary artery SMCs in culture [11,12,17,18]. The physiological function and molecular composition of SOC channels is highly variable depending on cell type, therefore, the study of SMCs from different vascular beds across species is imperative to our understanding of the ramifications of SOCE in health and disease. We are the first to demonstrate PDGF-BB-augmented SOCE in growth-arrested, differentiated RASMCs. Our results illustrating increased SR Ca^{2+} release during CPA exposure in the absence of extracellular Ca^{2+} and increased SOCE following the reintroduction of extracellular Ca^{2+} in PDGF-BB treated RASMCs are similar to those seen in pulmonary artery SMCs [11,18]. The presence of nifedipine in all solutions and the ability to block Ca^{2+} entry with Gd^{3+} , a well known inhibitor of SOCE [15,26], clearly identify the response as store-operated.

While recent research has begun to solidify increased SOCE as a cellular response to vascular injury and disease in proliferating SMCs, identification of the molecular mechanisms mediating SOCE has remained controversial. Recent attention has focused on the role of the Ca^{2+} -independent phospholipase A_2 (iPLA $_2$, also known as PLA $_2$ Group VI) as a regulatory mechanism in SOC influx. The products of iPLA $_2$ activation, lysophospholipids and arachidonic acid, have been shown to activate and inhibit SOCE, respectively [15,38-41]. Several studies have also shown measures of SOCE to be sensitive to the irreversible iPLA $_2$ inhibitor bromoenol lactone (BEL) in several cell types [38-46]. Our data provide further support for this mechanism of SOCE regulation in RASMCs as Figure 2 clearly illustrates the inhibition of Ca^{2+} influx (as indicated by Mn^{2+} quench) by 25 μ M BEL.

Interestingly, modulation of RASMC phenotype also appears to be linked to a BEL-sensitive mechanism. Although it is becoming apparent that alterations to SMC phenotype is a hallmark feature of the vascular response to disease, repair, and regeneration, the molecular signaling regulating this process has not been fully elucidated. Treatment with PDGF-BB is associated with the downregulation of SMC-specific marker genes indicative of a dedifferentiated phenotype, including smooth muscle myosin heavy chain (SMMHC), smooth muscle α -actin, and smoothelin [4,6,18,24,34-37,47-50]. More recently, the upregulation of $K_{Ca3.1}$ has also been shown following PDGF-BB treatment and implicated in the mediation of SMC phenotype modulation [6]. In the current study, we show for the first time inhibition of PDGF-BB-induced modulation of SMC phenotype by co-treatment with BEL. Upregulation of $K_{Ca3.1}$ and downregulation of SMMHC mRNA were completely blocked by BEL, implicating an iPLA $_2$ -mediated mechanism of PDGF-BB-induced SMC phenotype modulation. BEL also inhibited PDGF-BB induced downregulation of myocardin, a serum response factor (SRF) co-activator required for the transcription of SMC-specific marker genes dependent on the CC(A/T) $_6$ GG (CARg) promoter element, including SMMHC [4,5,17,24,51]. Interestingly, exposure to BEL stimulated mRNA expression of both myocardin and SMMHC in both CNT and PDGF-BB treated RASMCs *in vitro*, indicating the potential involvement of iPLA $_2$ in the basal regulation of these genes. To further test the involvement of iPLA $_2$ in SMC phenotypic

modulation, experiments were also conducted in the presence of another iPLA₂ inhibitor, methyl arachidonyl fluorophosphonate (MAFP). MAFP attenuated but did not fully inhibit PDGF-BB augmented K_{Ca}3.1 expression and did not inhibit PDGF-BB induced downregulation of SMMHC or myocardin. Although BEL is commonly used as an irreversible inhibitor of iPLA₂, it also inhibits phosphatidic acid phosphohydrolase-1 (PAP-1), a Mg²⁺-dependent enzyme which catalyzes the conversion of phosphatidic acid to diacylglycerol (DAG) [28-30]. The failure of MAFP to recapitulate the effects of BEL indicates a potential interaction between the two mechanisms. Active PLA₂ and its metabolites, including arachidonic acid, activate Ras/MAP kinase signaling pathways while DAG is known to promote IP₃ and PKC activation [52,53]. PKC activation via PAP-1 produces DAG, which is known to stimulate Fos/Jun heterodimers that bind to AP-1 [54], a transcriptional complex demonstrated to regulate the K_{Ca}3.1 promoter [54,55]. Therefore, inhibition of both iPLA₂ and PAP-1 by BEL may be responsible for the complete inhibition of PDGF-BB induced K_{Ca}3.1 upregulation demonstrated in Figure 3, whereas inhibition of iPLA₂ alone by MAFP resulted in only partial inhibition of PDGF-BB induced K_{Ca}3.1 upregulation (Fig. 4). Future studies are necessary to fully elucidate the BEL-sensitive signaling mechanisms involved in the regulation of PDGF-BB-induced SMC phenotype modulation.

Previous evidence was lacking as to whether increased K_{Ca}3.1 mRNA expression is dependent on PDGF-BB enhanced SOCE. Injury and mitogen-augmented increases in SOCE have been identified as integral to proliferation in a variety of SMC types [12,18,19], however, less is known in regards to its role in SMC phenotype modulation. Recent studies have shown Ca²⁺ entry through voltage-dependent or store-operated Ca²⁺ channels can influence gene expression in SMCs via Ca²⁺/cAMP response element binding protein and Ca²⁺/calmodulin kinase/calcineurin-dependent pathways [20-23]. Our laboratory has previously outlined the potential involvement of the AP-1 transcriptional complex in the upregulation of K_{Ca}3.1 by PDGF-BB [6,10,54,55] and enhanced SOCE in human pulmonary artery endothelial cells has been shown to augment AP-1 DNA binding activity [10]. Here, we provide the first evidence that modulation towards a de-differentiated phenotype by PDGF-BB, i.e. upregulation of K_{Ca}3.1 and suppression of SMC marker genes, is not dependent on SOCE. Treatment with the SOCE blocker Gd³⁺ or chelating of extracellular Ca²⁺ with EGTA did not inhibit PDGF-BB induced K_{Ca}3.1 upregulation or SMMHC and myocardin downregulation indicating that the initial modulation of gene expression occurs independent of increases in Ca²⁺ entry through store-operated mechanisms.

In conclusion, our results provide novel evidence that mitogen-induced modulations in RASMC phenotype and SOCE are BEL-sensitive. These observations support the conclusions of others [38,39,41-45] and provide a novel mechanism for SMC phenotype modulation by iPLA₂ and/or PAP-1. Despite a common iPLA₂ and/or PAP-1 dependence, suppression of SMC-specific gene expression appears to occur independent of SOCE as mitogen induced modulation of RASMC phenotype was not dependent on SOCE. Delineation of the iPLA₂ and/or PAP-1 dependent mechanisms regulating K_{Ca}3.1 and SMC phenotype should aid in the design of future treatment strategies targeting vascular remodeling and may prove useful in limiting atherosclerosis and/or post-stent restenosis.

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Fig. 1.

PDGF-BB increases intracellular Ca^{2+} following depletion of SR Ca^{2+} stores. A, Representative traces from CNT and PDGF-BB treated RASMCs illustrating mitogen augmented increases in SOCE. B, Summarized data from all experiments. CPA increased peak $[Ca^{2+}]_{intra}$ in cells treated with PDGF-BB (CPA MAX- paired samples t-test, $*P < 0.01$ vs. PDGF-BB Baseline1; independent samples t-test, $*P < 0.01$ vs. CNT CPA MAX). PDGF-BB increased peak $[Ca^{2+}]_{intra}$ to a greater extent after 2 mM Ca^{2+} intra was added following SR Ca^{2+} depletion (Ca^{2+} MAX-independent samples t-test, $^{\dagger}P < 0.05$ vs. CNT Ca^{2+} MAX). The addition of 10 μ M Gd^{3+} reduced $[Ca^{2+}]_{intra}$ in both groups (Gd^{3+} -paired samples t-test, $*P < 0.01$ vs. PDGF-BB & CNT Ca^{2+} MAX). The change in $[Ca^{2+}]_{intra}$ from Baseline2 to Ca^{2+} MAX was greater in PDGF-BB treated SMCs (Bar graph-independent samples t-test, $*P < 0.01$ vs. CNT)

Fig. 2.

PDGF-BB increases rate of SOCE. A, Representative traces demonstrating increased rate of F_{360} quench by Mn^{2+} in PDGF-BB treated cells. Pretreatment with 25 μM BEL completely inhibits F_{360} quench by Mn^{2+} in PDGF-BB treated RASMCs. B, Rate of Mn^{2+} influx is significantly greater in PDGF-BB treated SMCs, indicative of an increase in rate of SOCE (CPA- independent samples t-test, $*P < 0.05$ vs. CNT). BEL completely inhibited Mn^{2+} influx in both CNT and PDGF-BB (BEL). Mn^{2+} influx rate was not elevated when SR Ca^{2+} stores were not depleted (NO CPA).

Fig. 3.

BEL inhibits PDGF-BB induced RASMC phenotype modulation. A, Upregulation of $K_{Ca}3.1$ by PDGF-BB is inhibited by BEL (2×2 ANOVA- PDGF-BB & BEL main effect, PDGF-BB $\times$ BEL interaction; $*P < 0.01$, simple effect vs. CNT). B-C, BEL increases SMMHC and myocardin expression (2×2 ANOVA- BEL main effect). The effect of PDGF-BB on SMMHC and myocardin expression is dependent on BEL (2×2 ANOVA-PDGF-BB $\times$ BEL interaction; simple effects- $*P < 0.05$ vs. 8 hour +PDGF-BB).

Fig. 4.

MAFP attenuates PDGF-BB induced upregulation of $K_{Ca3.1}$ but has no effect on SMMHC or myocardin expression. A, The $iPLA_2$ inhibitor MAFP attenuated upregulation of $K_{Ca3.1}$ by PDGF-BB (2×2 ANOVA- PDGF-BB & MAFP main effect, PDGF-BB $\times$ MAFP interaction; $*P < 0.01$, simple effect vs. CNT & PDGF-BB + MAFP). B-C, PDGF-BB and MAFP induced downregulation of SMMHC and myocardin mRNA expression at 8 HR (2×2 ANOVA- PDGF-BB & MAFP main effect). Although PDGF-BB induced downregulation of SMMHC was not dependent on MAFP ($*P < 0.05$, simple effect vs. +PDGF-BB), their combined effects reduced myocardin expression to a greater extent (PDGF-BB $\times$ MAFP interaction; $*P < 0.01$, simple effect vs. +PDGF-BB).

Fig. 5.

SOCE is not required for PDGF-BB induced phenotype modulation in RASMCs. A, Blockade of SOCE with 10 μ M Gd³⁺ did not inhibit upregulation of K_{Ca}3.1 by PDGF-BB (2 $\times$ 2 ANOVA- PDGF-BB main effect; *P < 0.01, simple effect vs. CNT) and Gd³⁺ increased K_{Ca}3.1 expression alone and enhanced the effect of PDGF-BB at 8 HR (2 $\times$ 2 ANOVA- Gd³⁺ main effect, PDGF-BB $\times$ Gd³⁺ interaction; simple effects- †P < 0.05 vs. CNT). B-C, PDGF-BB-induced decreases in SMMHC and myocardin expression were not attenuated by co-treatment with Gd³⁺ (2 $\times$ 2 ANOVA- PDGF-BB main effect; *P < 0.01, simple effect vs. +PDGF-BB & PDGF-BB + Gd³⁺).

Fig. 6. Buffering of extracellular Ca^{2+} does not inhibit phenotype modulation by PDGF-BB. A, Buffering $[Ca^{2+}]_{extra}$ with EGTA did not prevent PDGF-BB induced upregulation of $K_{Ca3.1}$ (2×2 ANOVA- PDGF-BB main effect; * $P < 0.01$, simple effect vs. CNT). B-C, PDGF-BB reduces SMMHC and myocardin expression at 8 HR in the presence of EGTA (2×2 ANOVA- PDGF-BB main effect; * $P < 0.05$, simple effect vs. +PDGF-BB).

Table 1

Primer sequences (5' to 3')

Target Gene	Forward Primer	Reverse Primer
SMMYHC	CAG TTG GAC ACT ATG TCA GGG AAA	ATG GAG ACA AAT GCT AAT CAG CC
K _{Ca} 3.1	CTG AGA GGC AGG CTG TCA ATG	ACG TGT TTC TCC GCC TTG TT
Myocardin	CGG ATT CGA AGC TGT TGT CTT	AAA CCA GGC CCC CTT CC
iPLA ₂	TGG AAT TAA GCA GGC GGC AGA AAC	AAG ATA AGG CGC CTC CCT GAG AAA
18S	CGG CTA CCA CAT CCA AGG AA	AGC TGG AAT TAC CGC GGC