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The non-excitable smooth muscle: Calcium signaling and phenotypic switching during vascular disease

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Abstract

Calcium (Ca^{2+}) is a highly versatile second messenger that controls vascular smooth muscle cell (VSMC) contraction, proliferation, and migration. By means of Ca^{2+} permeable channels, Ca^{2+} pumps and channels conducting other ions such as potassium and chloride, VSMC keep intracellular Ca^{2+} levels under tight control. In healthy quiescent contractile VSMC, two important components of the Ca^{2+} signaling pathways that regulate VSMC contraction are the plasma membrane voltage-operated Ca^{2+} channel of the high voltage-activated type (L-type) and the sarcoplasmic reticulum Ca^{2+} release channel, Ryanodine Receptor (RyR). Injury to the vessel wall is accompanied by VSMC phenotype switch from a contractile quiescent to a proliferative motile phenotype (synthetic phenotype) and by alteration of many components of VSMC Ca^{2+} signaling pathways. Specifically, this switch that culminates in a VSMC phenotype reminiscent of a non-excitable cell is characterized by loss of L-type channels expression and increased expression of the low voltage-activated (T-type) Ca^{2+} channels and the canonical transient receptor potential (TRPC) channels. The expression levels of intracellular Ca^{2+} release channels, pumps and Ca^{2+}-activated proteins are also altered: the proliferative VSMC lose the RyR3 and the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase isoform 2a pump and reciprocally regulate isoforms of the ca^{2+}/calmodulin-dependent protein kinase II. This review focuses on the changes in expression of Ca^{2+} signaling proteins associated with VSMC proliferation both in vitro and in vivo. The physiological implications of the altered expression of these Ca^{2+} signaling molecules, their contribution to VSMC dysfunction during vascular disease and their potential as targets for drug therapy will be discussed.

Keywords

Vascular smooth muscle cell proliferation; Phenotypic switching; Ca^{2+} signaling; Canonical transient receptor potential channels; L-type and T-type voltage-dependent Ca^{2+} channels; Membrane potential; Potassium channels; Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)

Introduction

Vascular smooth muscle cells (VSMC) are one of the major cell types of the blood vessel wall and are instrumental in the maintenance of blood vessel integrity and the control of vascular tone [157]. Calcium (Ca^{2+}) signaling plays a central role in VSMC cell function. Ca^{2+} can

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originate from two distinct sources, entry from the extracellular milieu through plasma membrane channels or release from internal stores, namely the sarcoplasmic reticulum (SR). It is widely accepted that a global rise in cytoplasmic Ca^{2+} concentration leads to VSMC contraction [155]. The contractile characteristics of VSMC are quite different from those of cardiac myocytes. Unlike contractions of cardiac myocytes which are rapid and of relatively short duration, VSMC are characterized by relatively slow, in some cases sustained and tonic contractions. In vivo, VSMC contractility is subject to influences from several neuronal, humoral, and endothelial factors; they are partially constricted with resting Ca^{2+} concentrations around 100–300 nM, several orders of magnitude lower than the extracellular space [147].

Excitation–contraction coupling

VSMC membrane potential is critical for the control of cytoplasmic Ca^{2+} and therefore the maintenance of the vascular tone. Depolarization of VSMC plasma membrane activates the L-type high voltage-gated Ca^{2+} channels ($Ca_v1.2$) at the plasma membrane leading to an increased Ca^{2+} entry [30]. This global rise in intracellular Ca^{2+} leads to the initiation of the contractile response through activation of Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK). In turn, Ca^{2+}, through the process of Ca^{2+}-induced Ca^{2+} release (CICR) [117], activates the Ryanodine receptor (RyR) leading to Ca^{2+} release from the SR [152]. Unlike cardiac muscle, where the Ca^{2+} entry is further amplified through CICR, this process seems to be locally restricted to few subplasmalemmal Ca^{2+} sparks sites in VSMC [68]. Paradoxically, Ca^{2+} sparks via RyR in VSMC negatively regulate contraction through activation the large conductance Ca^{2+}-activated potassium (K^{2+}) channels ($K_{Ca}1.1$; also known as BK_{Ca}) leading to outward K^+ currents, subsequent hyperpolarization, decrease Ca^{2+} entry through L-type Ca^{2+} channels and VSMC relaxation [95]. Thus, the physiological effects (vasoconstriction versus vasorelaxation) of Ca^{2+} in VSMC are determined by the spatial distribution and the magnitude (global versus local) of the Ca^{2+} signal [55,95,152]. Reduced global Ca^{2+} levels and subsequent VSMC relaxation might also be achieved through extrusion or uptake of cytoplasmic Ca^{2+} by the plasma membrane Ca^{2+}-ATPase (PMCA) and the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pumps, respectively.

PLC-coupled receptors and receptor-operated channels

In addition, the contractility of VSMC in vivo is under the control of a plethora of vasoactive agonists (e.g., angiotensin II, vasopressin, endothelin) that exert their function through binding to membrane receptors that usually couple to phospholipase C (PLC) isoforms to stimulate Ca^{2+} release through the inositol-1,4,5-triphosphate (IP_3) receptor (IP_3R) [15] and concomitant Ca^{2+} entry via store-operated (SOC) [104,113] and receptor-operated Ca^{2+} (ROC) [10,19, 104] channels[1]. These channels conduct ions according to their electrochemical gradient across the plasma membrane, are operational within resting membrane potential (approximately −60 mV), and are inhibited by membrane depolarization. The exact contribution of PLC-coupled receptor activation to global Ca^{2+} levels during EC-coupling is still a matter of debate. However, one can imagine a scenario where discrete Ca^{2+} entry through ROC channels—or for that matter the generation of lipid second messengers that follow PLC-coupled receptor ligation—could affect the activation of chloride (Cl^-) and/or K^+ channels to alter the membrane potential and induce the activation of L-type Ca^{2+} channels. It has long been proposed that changes in membrane potential may occur as a result of influx of ions through ROC channels [130]. For example, activation of the non-selective receptor-activated canonical transient

[1]SOC channels can be considered a type of ROC channels. SOC channels are physiologically activated by the depletion of internal Ca^{2+} stores through IP_3-mediated Ca^{2+} release, and also by pharmacological agents that deplete the internal Ca^{2+} stores without increasing the levels of IP_3 such as the SERCA pump inhibitor thapsigargin. ROC channels include channels that are activated by signaling mechanisms downstream of membrane receptors, including second messengers such as IP_3, diacylglycerol (DAG) and arachidonic acid (SMOC channels), Ca^{2+}, cyclic nucleotides etc. In fact, the use of the ROC nomenclature which lumps together diverse ionic conductances is a reflection of our ignorance of the molecular identity and the exact mechanisms of activation of these ion channels.

receptor potential 6 (TRPC6) channel (discussed in detail later in this review) in VSMC was suggested to contribute to membrane depolarization and subsequent L-type Ca^{2+} channel activation [128,153]. Although nonselective cation channels such as TRPC6 conduct large sodium (Na^+) currents and thus can potentially depolarize the plasma membrane, the exact mechanisms by which vasoactive factors induce depolarization remain poorly understood. Even more confusing is the suggestion that a localized Ca^{2+} signal can couple to either contraction or relaxation and that this coupling is, to a large extent, determined by the specific ion pumps and channels contained within the plasma membrane–SR junctional complexes [77]. For example, the Ca^{2+} release through the IP_3R could conceptually, depending on the spatial properties of the Ca^{2+} signal, either cause VSMC contraction or relaxation; VSMC contraction would occur by a global IP_3-mediated Ca^{2+} rise while spatially restricted IP_3-mediated Ca^{2+} signals that are functionally equivalent to RyR-generated local subplasmalemmal Ca^{2+} sparks would cause relaxation. Studies by Bayguinov et al. have suggested such a differential regulation of VSMC contractility by PLC-coupled agonists [11, 12]. These authors showed that IP_3-mediated Ca^{2+} signals are not only cell type dependent but that within VSMC from the same vascular bed different PLC-coupled agonists can cause either contraction or relaxation depending on their differential regulation of membrane potential through Ca^{2+}-dependent activation of K^+ channels [11,12]. For a comprehensive review on spatially localized elementary Ca^{2+} signals, oscillations, and gradients and how they shape the specificity of Ca^{2+}-mediated responses in VSMC, the reader is referred to these review articles [16,77].

Evidently, Ca^{2+} dynamics during the excitation–contraction coupling (EC coupling) in VSMC are more complex with spatially and temporally coordinated events orchestrated by additional players, including but not limited to, mechano-sensitive Ca^{2+} entry channels, the sodium/calcium exchanger (NCX), K^+ and Cl^- channels. Furthermore, the impact of these Ca^{2+} signals extends beyond the short-term EC coupling to long-term effects through activation of transcription factors to induce profound changes in gene expression patterns, including those of contractile proteins and Ca^{2+} handling proteins themselves. A thorough discussion of this excitation–transcription coupling (ET coupling) is beyond the scope of this paper, and the reader is referred to the recent review articles by Owens, Lounsbury and their colleagues [9, 147].

Smooth muscle phenotypic switching

Smooth muscle provides not only structural integrity for the vessel wall but also precise regulation of vascular tone and blood pressure [101,102]. A striking feature of smooth muscle is the considerable cellular heterogeneity apparent within the media of the vessels. The presence of phenotypically distinct VSMC populations within the vessel wall is generally hypothesized to be important in the plasticity characteristic of vascular smooth muscle [90,101,102]. In response to various environmental stimuli, including growth factors, cytokines, mechanical influences, and various inflammatory mediators, the resident quiescent VSMC undergo transcriptional changes affecting both the downregulation of contractile proteins and concurrent upregulation of proteins supporting a proliferative phenotype [70,102,147]. This plasticity is believed to have evolved as a mechanism of vascular repair during injury and/or vascular adaptation to increasing demands by enabling VSMC to “switch” to a non-contractile, proliferative and migratory phenotype. This phenotypic switch is under tight control and is often reversible but under certain circumstances can largely contribute to vascular disease states such as atherosclerosis, hypertensive microvessels, vein graft failure, and restenosis following percutaneous intervention [31,123].

VSMC phenotypic switching during vascular disease is complex and most likely is the result of loss of humoral and endothelial factors or stimuli that sustain the differentiated state

combined with acquisition of pro-proliferative and promigratory signaling pathways such as those mediated by growth factors and cytokines. Most studies of VSMC phenotypic switching have focused on identification of factors that promote this process (e.g., growths factors and cytokines, oxidative stress, mechanical stress, endothelial factors) or on transcriptional regulation of phenotype-specific genes [70,101,102]. These studies have mainly focused on the loss of contractile proteins with very few reports devoted to the acquisition of pro-proliferative or pro-migratory proteins such as ion channels and pumps [14,32,74].

It is becoming increasingly apparent that a Ca^{2+} signaling pathway is responsible for transcriptionally regulating its own components whereby Ca^{2+} entry *via* a specific Ca^{2+} channel activates gene transcription and increased expression at the plasma membrane of that channel protein, as demonstrated for TRPC6 [75]. In a similar fashion, a general model has emerged in VSMC whereby Ca^{2+} signals controlled by large conductance K^+ channels $K_{Ca}1.1$, voltage-gated L-type Ca^{2+} channels and RyR, which are down-regulated in synthetic VSMC, couple to transcription of differentiated contractile protein markers [147]; and conversely, as will be discussed later in this review, signals resulting from activation of intermediate conductance Ca^{2+}-activated K^+ channels ($K_{Ca}3.1$) and TRPC channels, which are upregulated in synthetic VSMC, might couple to transcription of pro-proliferative protein markers. This review will focus on the changes in Ca^{2+} signaling proteins during VSMC phenotypic switch, including studies describing reciprocal regulation of CaMKII isoforms in proliferating VSMC and during vascular injury. The contributions of these changes to VSMC proliferation and the therapeutic potential of targeting components of Ca^{2+} signaling in the treatment of vascular disease will be discussed.

Loss of L-type and gain of T-type voltage gated Ca^{2+} channels

As stated earlier, L-type Ca^{2+} channels are important effectors in VSMC EC coupling as well as ET coupling [9,147]. L-type Ca^{2+} channels are characterized by high voltage-activated currents, large single-channel conductance and slow voltage-dependent inactivation [30]. L-type Ca^{2+} channels are subject to regulation by protein phosphorylation through second messenger-activated kinase pathways [30]. L-type Ca^{2+} channels are highly expressed in differentiated contractile or growth arrested smooth muscle cells where their presence correlates with the expression of smooth-muscle specific genes such as smooth muscle α-actin (SMα-actin) and smooth muscle myosin heavy chain (SM-MHC) [48]. Studies showed that Ca^{2+} entry through L-type Ca^{2+} channels are associated with activation of expression of the smooth muscle differentiation marker genes SMα-actin, SM-MHC and myocardin [147]. The pathways controlling the activation of these differentiation markers are dependent on the RhoA-Rho kinase (ROK) cascade [146], providing a novel signaling cascade in which physiological signals that couple excitation to contraction also contribute to transcriptional regulation of VSMC differentiation marker genes. The function of the T-type Ca^{2+} channel (named for their transient currents) has not been fully established, though several studies suggested their involvement in potentiating smooth muscle cell proliferation [34,118,119].

It has been demonstrated that the number of functional L-type Ca^{2+} channels was significantly decreased in dedifferentiated VSMC and increased upon differentiation with retinoic acid [48]. These authors found that the expression of the L-type Ca^{2+} channel α1 subunit directly correlates with the expression of SMα-actin and SM-MHC [48]. Subsequent studies provided evidence for the downregulation of the L-type Ca^{2+} channels during VSMC proliferation and in neointimal VSMC induced after balloon injury in rat aorta [63,114]. However, the expression of T-type Ca^{2+} channels was shown to increase in the VSMC synthetic phenotype [73,141], suggesting a pro-proliferative function of T-type Ca^{2+} channels in VSMC. What would be the functional consequence in VSMC for replacing the L-type voltage activated Ca^{2+} channel by another voltage activated Ca^{2+} channel, the T-type? Although increased expression of T-type

Ca^{2+} channels, which are activated by depolarization, in a proliferating cell that has lost its membrane excitability and its contractile proteins may seem paradoxical, it is worth mentioning that by comparison with L-type Ca^{2+} channels (threshold of activation −40 mV; full activation at 0 mV), T-type Ca^{2+} channels are characterized by activation at much more negative membrane potentials (20 to 40 mV more negative than the L-type) [91], rapid inactivation and small single-channel conductance [34]. Of special interest, the low voltage activation of T-type channels is particularly relevant for the synthetic VSMC, reminiscent of non-excitable cells, characterized by more hyperpolarized resting membrane potentials, at which T-type Ca^{2+} channels might preferentially operate. Indeed, electrophysiological recordings have determined that the resting membrane potential of VSMC in culture ranges from −45 to −70 mV, while membrane potentials of VSMC measured in vivo were −40 to −55 mV [6,49,91].

A different kind of switch, this time occurring within the L-type Ca^{2+} channel family, have been described in atherosclerotic plaques, where decreased expression of the $Ca_v1.2\alpha1$ transcript is accompanied by the appearance of a splice variant of the same channel where exon-21 is replaced with exon-22 [133]. Ca^{2+} currents through the exon-22 isoform recover from inactivation significantly faster than other isoforms, suggesting increased Ca^{2+} current densities. Interestingly, a 15-mV shift of the exon-22 channel activation curve to more negative potentials was observed which might contribute to proliferation by the increase of Ca^{2+} entry in VSMC at more hyperpolarized membrane potentials. In fact, Tiwari et al. [133] showed that inhibition of cell proliferation by serum deprivation in confluent VSMC monolayers completely eliminated the exon-22 isoform of the $Ca_v1.2\alpha1$ transcript, which was reversible upon stimulation of proliferation by addition of serum to nonconfluent cells.

Clearly, the role of L-type Ca^{2+} channels in vascular disease is complex, and their decreased expression during vascular injury is not common to all vascular diseases; this highlights the need of caution in extrapolating data from one vascular disease model to another. For instance, L-type Ca^{2+} channels are upregulated in mesenteric and skeletal arteries of spontaneously hypertensive rats where they seem to contribute to increased vascular tone [110]. By contrast, hypercholesterolemia-induced atherosclerosis is characterized by inhibition of activity (without change in expression) of L-type Ca^{2+} currents in the coronary circulation [24].

The idea that hyperpolarization-activated Ca^{2+} entry plays a role in VSMC proliferation while depolarization-activated Ca^{2+} channels are involved in VSMC EC-coupling is compatible with the increased expression, in the VSMC synthetic proliferative phenotype, of Ca^{2+} channels activated at more negative membrane potentials. This concept is supported further by the increased expression in the VSMC synthetic phenotype of TRPC channels that optimally operate at negative membrane potentials.

Increased expression of TRPC cation channels

TRPC channels are one of the major TRP families of nonselective cation channels and termed “canonical” because they are structurally the closest to the founding family member, *Drosophila* TRP [98,138,143]. The seven TRPCs share in common the property of activation through PLC-coupled receptors and were therefore proposed as components of native SOC and ROC channels in different cell types [138,140,143]. The TRPC family can be divided into four subfamilies: TRPC1, TRPC2, TRPC3/6/7, and TRPC4/5 (or, TRPC1 can be included with TRPC4 and -5). TRPC2 is a pseudogene in humans but encodes a functional channel in most other mammals.

The discovery of TRPC channels in the vasculature led to the hypothesis that TRPC channels were the long-sought after vascular version of SOC channels [53]. For example, all of the seven family members (TRPC1-7) are expressed in the vasculature [64,97] and studies examining the function of TRPC1 in VSMC suggest they are linked to Ca^{2+} entry associated with

intracellular Ca^{2+} store depletion [7,13]. In no case has an expressed TRPC (which are nonselective) served to recapitulate the electrophysiological behavior of the archetypical Ca^{2+} selective SOC channel, CRAC found in hematopoietic cells (lymphocytes, mast cells) [104]. However, SOC current measurements in VSMC have been shown to be non selective and to present distinct electrophysiological features than those of CRAC [20]. Thus, it is conceivable that TRPC channels do function physiologically as SOC in VSMC, where they are more likely to form subunits of less Ca^{2+} selective SOC.

Strong evidence supports the notion that ectopically expressed TRPC channels are primarily regulated by receptor-operated mechanisms, as in most cases (but not all, TRPC1 being a notorious exception) TRPC members are activated subsequent to stimulation of receptors that activate different isoforms of PLC, in a manner independent of store depletion [138]. Under these expression conditions, it is believed that TRPC channels predominantly form homotetramers and it is clearly established that under these conditions TRPC family members 3, 6, and 7 are activated by DAG in a PKC-independent manner [56,99,139], while family members 1, 4, 5 are completely unresponsive to DAG activation and their exact mechanisms of activation remain elusive. It is worth mentioning that the idea that TRPC channels operate as SOC or ROC does not have to be mutually exclusive as TRPC members may form heteromultimeric channels in native tissues [57], and different subunit stoichiometry may confer the activities characteristic of either a SOC or ROC [112]. Furthermore, recent studies have reported protein–protein interactions between TRPC channels and the newly discovered Stim1 and Orai1 proteins [80,100], which were clearly demonstrated to be respectively the ER calcium sensor and the pore forming unit of the archetypical Ca^{2+} selective SOC channel, CRAC [42,81,121,145,164,165]. A role for TRPC involvement in different vascular diseases arises from extensive cell culture and whole animal studies [97]. The most commonly expressed TRPC isoforms in the vasculature are TRPC1, 4, and 6. TRPC 3, 5, and 7 isoforms have also been detected, though to a lesser extent and less frequently [64]. Only low levels of TRPC2 expression have been detected in the vasculature, and its function at this time remains unknown [25].

As stated earlier, TRPC channels (and ROC channels in general) are activated downstream of PLC, allow Ca^{2+} into the cells at negative membrane potentials and are inhibited by depolarization. In VSMC in culture and in models of VSMC injury as well as in other cases of vascular disease, TRPC channel expression dramatically increases. Specifically, the loss in contractile phenotype in proliferative VSMC in culture correlated with over fivefold increase in TRPC1 and TRPC6 mRNA and protein expression and increased whole cell Ca^{2+} currents [14]. In the same study, balloon dilatation of human internal mammary artery showed a similar increase in TRPC1 and TRPC6 mRNA expression 48 h post injury [14]. TRPC1 is upregulated following experimental vascular injury in mice and pigs, and is associated with the conversion of smooth muscle cells from a quiescent to a proliferative phenotype initiating neointimal formation [74]. Upregulation of TRPC1 was associated with enhanced Ca^{2+} entry and cell cycle activity. The functional relevance of TRPC1 upregulation in neointimal growth was studied in a human saphenous vein organ culture where TRPC1 function was blocked with a specific antibody targeted to its third extracellular loop (E3); this approach significantly inhibited neointimal growth in organ culture as well as calcium entry and VSMC proliferation [74].

In pulmonary artery SMC, TRPC1 and TRPC6 expression increases following serum stimulation [50,160]. ATP-induced mitogenesis in pulmonary artery SMC was shown to be mediated through CREB-induced TRPC4 gene transcription [162]. The in vivo relevance of TRPC isoform upregulation in human pulmonary artery SMC was observed in patients with idiopathic pulmonary arterial hypertension (IPAH), a disease characterized by excessive pulmonary artery SMC proliferation [43]. In these patients, TRPC3 and TRPC6 were strongly

upregulated. In pulmonary artery SMC cultured from patients with IPAH siRNA directed against TRPC6 markedly attenuated proliferation [158], suggesting TRPC channel overexpression might be important for increased proliferation during IPAH. Pulmonary hypertension caused by chronic hypoxia also involves excessive proliferation as well as contraction of pulmonary artery SMC. In a rat model of pulmonary hypertension, TRPC1 and TRPC6 upregulation was shown to require the expression of hypoxia inducible factor 1 (HIF-1) [149,150]. TRPC channel perturbation is also proposed to be a factor involved in essential hypertension, as spontaneously hypertensive rats expressed abnormally high levels of TRPC3 compared to wild type control animals [84].

Loss of $K_{Ca}1.1$ and gain of $K_{Ca}3.1$ potassium channels

Ca^{2+}-activated K^+ channels indirectly regulate the flow of Ca^{2+} ions into cells by modulating the membrane potential through K^+ efflux. Quiescent contractile VSMC express $K_{Ca}1.1$ (also called BK_{Ca}), large conductance K^+ channels that are activated by intracellular Ca^{2+} and whose activity increases in response to membrane depolarization. Efflux of K^+ through $K_{Ca}1.1$ causes hyperpolarization and therefore limits extracellular Ca^{2+} entry through the L-type voltage gated Ca^{2+} channels [25,76,95]. In this capacity, $K_{Ca}1.1$ serves as a negative feedback regulator that controls intracellular Ca^{2+} concentration and VSMC contraction.

Interestingly, $K_{Ca}1.1$ is downregulated when VSMC switch from a contractile to synthetic phenotype. Loss of $K_{Ca}1.1$ has previously been observed both in synthetic VSMC in culture and following balloon injury in the rat carotid artery [72,96]. Reciprocally, synthetic or proliferating VSMC upregulate the intermediate conductance Ca^{2+}-activated K^+ channel protein 4 ($K_{Ca}3.1$), encoded by the *KCNN4* gene [96]. Importantly, $K_{Ca}3.1$ blockade with a selective inhibitor, TRAM-34 inhibited neointimal formation by 40% after balloon-catheter induced injury in the rat, suggesting that $K_{Ca}3.1$ upregulation following vascular injury is functionally significant in proliferating VSMC [72]. A subsequent study showed that inhibition of $K_{Ca}3.1$ suppresses neointimal formation in the human saphenous vein, a vessel isolated from patients undergoing coronary artery bypass surgery and prone to failure as a result of neointimal hyperplasia [32].

As discussed above for Ca^{2+} channels, here again the most important difference between $K_{Ca}1.1$ and $K_{Ca}3.1$ channels appears to be their voltage dependence. Indeed, unlike $K_{Ca}1.1$ channels which require depolarization to optimally operate, $K_{Ca}3.1$ channels can open at negative membrane potentials which lead to further hyperpolarization of the cells. Thus, K^+ efflux through $K_{Ca}3.1$ can potentially continue to hyperpolarize the cells up to a membrane potential of approximately −80 mV (value of K^+ equilibrium potential) and subsequently provide additional driving force for robust Ca^{2+} entry through TRPC channels[2]. It appears therefore that $K_{Ca}3.1$ and TRPC (and possibly T-type Ca^{2+} channels) increased expression in synthetic SMC are functionally connected and represent components of the same Ca^{2+} signaling unit, important for increased Ca^{2+} entry at more hyperpolarized membrane potentials in VSMC. According to this paradigm, Ca^{2+} entry through hyperpolarization-activated channels might preferentially connect to downstream effectors necessary for promoting the proliferative and migratory capabilities of synthetic VSMC.

[2]Some TRPC channels such as TRPC3 and TRPC7 are known to possess considerable constitutive activity [38], and it was clearly demonstrated that growth factors such as the epidermal growth factor (EGF) can increase the membrane expression of constitutively active TRPC channels through increased vesicular trafficking [18,127]. Thus, it is reasonable to assume that strong hyperpolarization in VSMC would lead to increased constitutive activity of TRPC channels, and increased basal levels of cytoplasmic Ca^{2+}.

Loss of RyR3 Ca^{2+} release channels

RyR are Ca^{2+} release channels located in the membrane of the SR that are sensitive to Ca^{2+}, caffeine and the plant alkaloid ryanodine. Opening of RyR can cause local Ca^{2+} transients termed Ca^{2+} sparks that regulate the membrane potential through activation of K^+ channels [65,66]. RyR are structurally and functionally analogous to IP_3R, although they are approximately twice as large and have twice the conductance of IP_3R [21]. Similar to IP_3R, RyR are sensitive to cytosolic Ca^{2+} concentrations: nM to μM Ca^{2+} concentrations causing channel activation and μM to mM Ca^{2+} concentrations inhibiting channel function [21]. Also similar to the dual regulation of the IP_3R, RyR phosphorylation by the second messenger cyclic ADP ribose (cADPR) and protein kinase A (PKA) enhance Ca^{2+} sensitivity while phosphorylation by protein kinase C (PKC) decreases RyR Ca^{2+} sensitivity [65,83].

There are presently three molecularly distinct subtypes of RyR channels, RyR1, RyR2, and RyR3. While RyR channels are found in all muscle types, the vasculature appears to show a differential distribution of the three RyR isoforms [65,122]. For example, rat cerebral arteries and portal vein myocytes express all three isoforms [122], while mouse myometrial smooth muscle and rat aorta express mainly the RyR3 isoform [122,141]. It has been proposed that the RyR3 isoform is the prominent ryanodine receptor in smooth muscle [65]; however, the role of RyR3 in the vasculature is difficult to elucidate in that $RyR3^{-/-}$ mice do not differ in their spatial–temporal characteristics of Ca^{2+} sparks compared to control animals [122].

The in vitro conversion of aortic VSMC from the quiescent contractile state to the synthetic proliferating phenotype coincides with a loss in RyR3 expression and subsequent loss of the CICR mechanism [141]. The functional impact of loss of RyR3 in the intact aorta would not allow the vessel to oppose vasoconstriction and might possibly be involved in the hypersensitivity reaction to vasoactive substances observed in the pathology of hypertension and atherosclerosis. The expression levels of RyR during vascular injury have not been studied and the role of RyR in VSMC phenotypic switching and proliferation is unclear. While RyR function was not impaired in vivo in the aorta of hypercholesterolemic $ApoE^{-/-}$ mice in one study [142], a separate in vitro study did find that oxidized LDL induced a decrease in VSMC RyR channel density which resulted in a defect in the cells ability to regulate cytosolic Ca^{2+} [89]. As mentioned earlier, Ca^{2+} release through RyR is involved in vasodilatation of VSMC, and therefore suppression of this release pathway would alter the control of VSMC contractility. However, in vascular pathologies where VSMC lose their contractile proteins, it is reasonable to suggest that proliferation, rather than contractility, is likely responsible for the thickening of the vessel wall. Clearly, the downregulation of RyR3 represents one component (in addition to $K_{Ca}1.1$ and the L-type Ca^{2+} channels) of the Ca^{2+} signaling unit controlling EC-coupling that is altered during VSMC phenotypic switching. However, whether the loss of RyR3 in the synthetic VSMC is functionally connected to the promotion of the proliferative or migratory phenotype remains unknown.

Role of IP_3R Ca^{2+} release channels

In quiescent smooth muscle, agonists that act through G protein-coupled receptors or receptor tyrosine kinases can induce intracellular Ca^{2+} signals through activation of PLC. PLC catalyzes the hydrolysis of phosphatidylinositol4,5-bisphosphate (PIP_2) to produce the intracellular messengers IP_3 and diacylglycerol (DAG). IP_3 is highly diffusible in the cytoplasm and activates specific receptors (IP_3R) on the sarcoplasmic reticulum membrane [15]. There are three isoforms of the IP_3R: IP_3R1, IP_3R2, and IP_3R3 with IP_3R1 being the most widely expressed of these three and found in most cell types. The binding of IP_3 to the IP_3R changes the conformation of the IP_3R causing its opening and Ca^{2+} release from the SR. The Ca^{2+} release mediated by IP_3 diffuses through the cytoplasm thereby exciting neighboring receptors

and providing the momentum necessary to generate a Ca^{2+} wave. IP_3R are also sensitive to Ca^{2+} and in order to avoid random triggering of regenerative Ca^{2+} waves, Ca^{2+} has a biphasic effect on the receptor. As the intracellular Ca^{2+} concentration increases, Ca^{2+} exerts positive feedback and facilitate IP_3R channel opening [17,21]. However, a further increase in cytosolic Ca^{2+} has the opposite effect and actually inhibits the channel opening [21,83]. Interestingly, evidence suggests that high concentrations of IP_3 may prevent Ca^{2+}-dependent inactivation of IP_3R [22], an observation which is potentially important in proliferating VSMC in disease states characterized by increased pro-proliferative growth factors and endothelial factors acting through the PLC/IP_3 pathway.

Increasing evidence is supporting a role for the IP_3R as an important determinant of VSMC proliferation. Exposure of freshly explanted cerebral arteries to fetal bovine serum elevated IP_3 and the frequency of Ca^{2+} waves which were subsequently required for VSMC proliferation [154]. Proliferation was inhibited by several IP_3 receptor antagonists (100 μM 2-APB, 10 μM xestospongin C, or 25 μM U-73122) which dramatically reduced outgrowth cell number compared with ryanodine-treated (10 μM) arteries [154]. However, these experiments should be interpreted with caution since the effects of these pharmacological inhibitors could be partially due to inhibition of plasma membrane Ca^{2+} channels. In fact, 2-APB, at much lower concentrations (30 μM) is a potent inhibitor of SOC channels and is also known to partially inhibit TRPC channels [137], while U-73122 acts upstream to inhibit PLC, and would thus consequently inhibit the activation of receptor-operated channels, including all TRPC channels. Nonetheless, in serum-stimulated VSMC, proliferation is associated with a sustained increase in intracellular Ca^{2+} partially due to enhanced excitability of IP_3R following binding of IP_3. In addition, a recent report showed a sixfold increase in the expression level of IP_3R1 as VSMC progressed from G0 to G1/S of the cell cycle [5]. This increase was mediated by the cell cycle-associated transcription factor c-Myb and was accompanied by increased intracellular Ca^{2+} at the G1/S transition [5].

Although in vivo studies have not directly examined IP_3R expression/activation during vascular disease states, a recent study carried out in aortic rings of apolipoprotein E-deficient mice which had not yet developed atherosclerotic lesions, implicated IP_3R as an important player during the progression of atherosclerosis. In this particular study, IP_3R-mediated Ca^{2+} release in response to phenylephrine and ATP was significantly increased in hyperocholesteremic but plaque-free apo$E^{-/-}$ mice compared to wild type mice [142]; apo$E^{-/-}$ mice displayed an increase in basal levels of cytosolic calcium [142]. In a similar manner, endothelin-1, which induces a Ca^{2+} release through IP_3R, produced a greater Ca^{2+} release in diabetic dyslipedemic swine carotid artery as compared to controls [78].

Loss of the SERCA2a isoform

SERCA maintains intracellular Ca^{2+} homeostasis in VSMC by pumping Ca^{2+} from the cytoplasm into the SR stores. The SERCA pump is encoded by a family of three genes located on separate chromosomes, SERCA1, 2, and 3 [44]. The SERCA isoform diversity is greatly increased by alternative splicing of the transcripts, resulting in more than ten different SERCA isoforms currently characterized in different tissues and developmental states [106]. SERCA isoforms differ in their affinity for Ca^{2+}, resistance to oxidative stress and modulation by phospholamban (PLN) and Ca^{2+}/calmodulin kinase II. The activity of SERCA2 is dependent on its interaction with PLN which is inhibitory in its dephosphorylated form. Phosphorylated PLN dissociates from SERCA2, thus activating the Ca^{2+} pump and SERCA2 activity is proposed to account for removal of >70% cytoplasmic Ca^{2+} [86].

It is clearly established that SERCA2a plays an important role in cardiac function. Earlier studies have shown that SERCA2a levels are decreased in human failing hearts and in several

animal models of cardiac hypertrophy [8,151]. The idea that SERCA2a expression might protect against cell proliferation, originated from studies where heterozygous mice with a null mutation in the SERCA2 gene develop cancers of the skin and the upper digestive tract [85], as well as cardiac hypertrophy [107]. Additional studies showed that mice in which SERCA2a has been replaced by SERCA2b develop dilated cardiac hypertrophy [144]. In contrast, cardiac-specific expression of SERCA2a in transgenic animals showed improved contractile function under basal conditions and pressure overload [94]. Furthermore, while only SERCA2b is present in proliferating BC3H muscle cells, SERCA1, SERCA2a, and SERCA2b are present in the quiescent differentiated cell variant BC3H1 [36].

Quiescent smooth muscle cells express two splice variants of the SERCA2 gene, 2a and 2b.Vallot et al., have shown that SERCA2a is repressed in vitro in VSMC during serum- and PDGF-induced proliferation [141]. Work by Lipskaia et al. showed that in proliferating VSMC, SERCA2a expression is downregulated, while SERCA2b expression remains unchanged [82]. When VSMC are growth arrested or in confluent cultures SERCA2a expression is regained, pointing to an important link between alteration in the expression of Ca^{2+}-handling proteins and cell proliferation. Interestingly, the same authors provided evidence that adenoviral overexpression of SERCA2a inhibits VSMC proliferation and subsequent neointima formation following balloon catheter-induced injury in the rat carotid artery [82]. Specifically, overexpression of SERCA2a in serum-stimulated VSMC inhibited proliferation through inactivation of calcineurin and its target transcription factor nuclear factor of activated T-cells (NFAT) resulting in lowering of Cyclin D1 levels and cell cycle arrest at the G1 phase [82]. The authors concluded that the delivery of SERCA2a to the injured media reversed the alterations in Ca^{2+} handling observed in proliferating VSMC, suggesting SERCA2a expression and activity are important in maintaining the non-proliferative phenotype of smooth muscle [82,83]. In addition, overexpression of SERCA2a in proliferating VSMC in culture reduced the amplitude and propagation of IP_3-evoked Ca^{2+} signals [82], supporting a role of IP_3-mediated Ca^{2+} signaling in VSMC proliferation.

Role of PMCA

The PMCA is a high-affinity Ca^{2+} pump dedicated to the removal of Ca^{2+} from the intracellular to the extracellular space. In a manner similar to SERCA, the PMCA is an ATP-driven pump which moves Ca^{2+} against its concentration gradient, thus functioning as an important system involved in maintaining low levels of cytoplasmic Ca^{2+}. In mammals, four separate genes encode for the major PMCA isoforms (PMCA1 through 4) and the various gene products and splice variants which arise thereof show different patterns of expression during development as well as in different tissue beds [28,52]. While each isoform and splice variant can be regulated by multiple kinases and protein–protein interactions, the main regulator of PMCA function is Ca^{2+}-bound Calmodulin (CaM) [131]. Not surprisingly, it appears that the most important splicing occurs in the CaM-binding domain. Alternative splicing at this site alters the ability of CaM to bind and activate the pump. PMCA1 and PMCA4 are present in almost all cells and have therefore been proposed as housekeeping pumps, while PMCA2 and PMCA3 have a more constrained distribution and are possibly endowed with more specialized functions [27,52,111].

In the vasculature, medial smooth muscle cells appear to express multiple isoforms of both PMCA1 and 4 [3,131] and cell lines from canine carotid artery or saphenous vein medial wall express PMCA1b, 4a and 4b [3]. The role of PMCA1 might be to repress VSMC proliferation as cells in the G1/S phase of the cell cycle experienced lower levels of PMCA1 mediated through repression by the transcription factor c-Myb [61,62]. Overexpression of PMCA1 or suppression of c-Myb in these cells resulted in a reduced rate of cell proliferation assessed by a fewer number of cells entering the S phase [4,62]. Whole animal studies in which c-Myb has

been inhibited suggest that loss of PMCA1 function has important consequences on pathologic VSMC proliferation [4].

However, a study showed that upon placement in culture, carotid artery and saphenous vein VSMC showed a time-dependent upregulation of PMCA4a in a PI3-Kinase dependent manner and increased cell proliferation. Reciprocally, inhibition of PMCA4a upregulation with Wortmannin inhibited cell proliferation [3]. These results may appear incompatible with the general concept whereby decreased PMCA1 and SERCA2a, increased TRPC channels and IP_3-mediated Ca^{2+} release contribute to the sustained increase in cytoplasmic Ca^{2+} levels that are necessary to drive cell proliferation. A possible explanation for this discrepancy might be that the newly synthesized PMCA4a molecules could be targeted to specific membrane subdomains and therefore not significantly affecting the pool of cytoplasmic Ca^{2+} associated with cell proliferation. Alternatively, heterologous expression studies of human PMCA4a and 4b showed that PMCA4a has 7- to 20-fold lower affinity for calmodulin and Ca^{2+} and a higher basal activity in the absence of calmodulin than PMCA4b [40]. This would suggest that PMCA4a is probably important for maintaining Ca^{2+} homeostasis under basal conditions and that it operates optimally to extrude Ca^{2+} only when very high levels of cytoplasmic Ca^{2+} concentrations are reached within the cells. In proliferating VSMC, PMCA4a overexpression could thus be compatible with increased cytoplasmic Ca^{2+} levels necessary to drive cell proliferation while, at the same time, maintaining the Ca^{2+} levels below a certain threshold to protect against cell death by apoptosis.

Other changes involving various ion channels

There are other ion channels that see their expression change during VSMC switching to a proliferative phenotype that seem to contribute to increased cytoplasmic Ca^{2+}. Zhang et al. [161] demonstrated an upregulation of NCX and enhanced Ca^{2+} entry via NCX reverse mode in pulmonary artery SMC from patients with idiopathic arterial hypertension. These authors previously showed that cultured human pulmonary artery SMC express high levels of NCX and that enhanced NCX activity in its reverse mode produced an increase in cytoplasmic Ca^{2+} and promoted proliferation [163].

Quignard et al. [115] found that the tetrodotoxin-sensitive voltage gated Na^+ channel was not expressed in freshly isolated myocytes from human coronary artery but both expression of these channels and large voltage gated Na^+ currents were detected when the cells were grown in culture [115]. A microarray study in lung tissues from patients with idiopathic and familial pulmonary arterial hypertension showed increased mRNA expression of the voltage gated Na^+ channel β1 subunit [45]. The physiological significance of increased Na^+ currents in proliferating cells remains a mystery. Platoshyn et al. [109] showed evidence that increased Na^+ currents does not directly influence membrane potential, intracellular Ca^{2+} release or proliferation in cultured human pulmonary artery smooth muscle. These authors speculated that increased Na^+ currents could contribute to activation of upregulated NCX in its reverse mode, leading to an increase of the bulk of cytoplasmic Ca^{2+} and enhanced cell proliferation [109].

As discussed earlier, K^+ channels control membrane potential which in turn regulates the flow of Ca^{2+} across the plasma membrane. Karkanis et al. [69] demonstrated increased inwardly rectifying K^+ channels (Kir) currents in proliferative compared with contractile VSMC and showed increased mRNA and protein expression of Kir2.1 in proliferative cells, which displayed a more negative membrane potential than contractile cells [69].

The expression profile of voltage gated K^+ (Kv) channels is also altered during the transition of VSMC from the contractile to the proliferating phenotypes. While Kv1 channel members were the main contributors to the Kv currents in the human uterine contractile phenotype,

proliferating VSMC upregulated Kv3.4 expression [92]; Kv3.4 is characterized by a rapid inactivation. Interestingly, selective blockade of Kv3.4 channels decreased VSMC proliferation [92]. Another study showed that pulmonary artery SMC proliferation is associated with a decrease in Kv1.5 currents [159]. Early studies by DeCoursey et al. [37] have proposed voltage gated K^+ channels as important players in T-cell mitogenesis. Subsequent studies implicated many isoforms of K^+ channels in normal and/or pathological cell proliferation in different cell types [103]. The exact mechanisms by which voltage gated K^+ channel might regulate proliferation in VSMC that have lost their electrical excitability (or for that matter in electrically non-excitable cells in general) remain poorly understood and may well involve regulation of membrane potential and/or cell volume.

Cl^- channels which are expressed in both the plasma membrane and intracellular organelles of cells play an important role in various cell functions, including ion homeostasis, cell volume regulation, transepithelial transport, and regulation of electrical excitability [67]. There is also evidence implicating ClC-3, a voltage gated Cl^- channels, in VSMC proliferation; different Cl^- channel blockers were shown to inhibit the proliferation of VSMC induced by endothelin-1 (ET-1) [156], and transient transfection of rat aortic VSMC with antisense oligonucleotide specific to ClC-3 caused an inhibition in ET-1-induced expression of ClC-3 protein and cell proliferation of VSMC [148]. ClC-3 expression was upregulated in pulmonary artery smooth muscle cells and cardiac myocytes from hypertensive mice and in response to inflammatory mediators such as ET-1, platelet-derived growth factor (PDGF), IL-1β and TNFα; ClC-3 increase was shown to confer increased viability and enhanced protection against cell death induced by reactive oxygen species [35]. Voltage gated Cl^- channels can induce VSMC depolarization; the Cl^- equilibrium potential in VSMC is approximately −26 mV, a range at which L-type Ca^{2+} channels can be effectively activated. However, how exactly increased ClC-3 expression might functionally affect the proliferation of VSMC that has lost L-type Ca^{2+} channels expression is not known. As pointed out by Guan et al., medial hypertrophy, a key feature involved in arterial remodeling during hypertension, is caused mainly by the enhancement of VSMC proliferation in response to vascular injury. Because cell proliferation must sometimes lead to increased cell volume, it is therefore not surprising that ClC-3 function is associated with VSMC proliferation during vascular disease [51]. However, the involvement of ClC-3 as a volume-regulated channel remains controversial, and findings from several groups, unable to consistently measure plasma membrane ClC-3 currents, suggested that ClC-3 can reach the plasma membrane only under some conditions of overexpression and provided evidence for the expression of ClC-3 in intracellular vesicles (see [67] and references therein). Whether ClC-3 increased expression in proliferating VSMC is associated with increased ClC-3 proteins at the plasma membrane or in intracellular vesicles and how this pertain to VSMC proliferation remains a mystery.

Decrease of CaMKIIγ and increase of CaMK IIδ$_2$

The Ca^{2+}/calmodulin (Ca^{2+}/CaM)-dependent protein kinase II (CaMKII) is a ubiquitously expressed and multifunctional serine/threonine kinase that mediate many of the downstream effects of Ca^{2+} signaling. CaMKII isoforms can associate to form either homo- or hetero-multimeric holoenzyme composed of 10–14 subunits. In mammals, CaMKII isoforms are encoded by four different genes (α, β, γ, δ) which share extensive sequence homology and structure [135]. CaMKII transcripts are in turn subject to extensive alternative splicing, resulting in kinases with variable domains and specific cellular functions. Although ubiquitously expressed, CaMKII isoforms have a distinct pattern of expression in different tissue beds. Distribution of three distinct α and seven distinct β splice variants are mainly limited to the central nervous system where they are estimated to comprise up to 1% to 2% of the total protein in the forebrain and hippocampus, respectively [41,135]. In the brain, CaMKII is critical for regulating synaptic and behavioral plasticity and possibly memory [33]. To date,

there are 13 recognized CaMKIIγ splice variants. Similarly, 15 splice variants have been identified for the δ gene [29]. Both CaMKIIγ and CaMKIIδ are ubiquitously expressed with the highest levels observed in peripheral tissue [134]. Many studies have particularly focused on CaMKII function in heart and vascular smooth muscle and implicated the involvement of CaMKII in various functions including the following: mediation of Ca^{2+}-dependent gene transcription [116], regulation of protein interactions involved in contractility and cell motility [105,108,120,132], and control of intracellular Ca^{2+} dynamics [87].

In the vasculature, differentiated VSMC express CaMKII γ and δ variants [124,125]. In these cells, CaMKII γ isoforms have been implicated in the control of the differentiated contractile function [71,88,120]. However, cultured VSMC predominantly express the δ_2 (also referred to as δ_C) isoform [124]. CaMKII δ isoforms couple to ERK1/2 signaling [1,47], and the δ_2 isoform was shown to specifically regulate VSMC migration [105,108]. Upregulation of CaMKII δ isoforms was shown to occur in models of cardiac hypertrophy and heart failure [23,166], as well as in regenerating skeletal muscle after injury [2].

Recently, House et al. have showed that following dispersion and primary culture of VSMC, a significant increase in CaMKII δ_2 protein and a significant decrease in CaMKII γ protein within 30 h of dispersion which coincided with VSMC phenotypic switching including increased DNA synthesis and cell proliferation [58]. Interestingly, this reciprocal regulation of CaMKII isoforms during phenotypic switching occurred even when cells were dispersed under low serum concentrations (0.4%) which do not support proliferation, suggesting that the CaMKII isoform switch (and possibly changes in expression of other molecular players) occurs irrespective of VSMC proliferation [58]. Attenuation of the initial upregulation of the CaMKII δ_2 isoform in primary cultured cells using small-interfering RNA resulted in decreased serum-stimulated DNA synthesis and cell proliferation [58], arguing for a specific role of CaMKII δ_2 in driving VSMC proliferation. In a follow up study, similar results were obtained with rat carotid arteries subjected to balloon angioplasty where a significant increase of CaMKII δ_2 and a significant decrease of CaMKII γ were observed within 3 days post-injury in both medial smooth muscle and adventitial fibroblasts [59]. In this model, the neointimal VSMC expressed primarily CaMKII δ_2; incubation of the injured vessels with adenovirus encoding siRNA against CaMKII δ isoforms prevented upregulation of CaMKII δ_2 in the media and adventitia, inhibited cell migration and proliferation and neointima formation [59].

What is the physiological significance of the switch in CaMKII isoform expression? CaMKII isoforms are capable of autoregulation through autophosphorylation which confers to the kinase the unique ability to maintain catalytic activity without bound Ca^{2+}/CaM [60,129]. This is particularly important for the memory of the kinase allowing continuous activation after intracellular levels of Ca^{2+} has returned to their basal state [126]. It is increasingly acknowledged that different sources, frequencies, amplitudes, and spatiotemporal distributions of Ca^{2+} signals may result in activation of different downstream effectors, different patterns of gene expression and thus lead to different physiological outcomes [154]. CaMKII isoforms are capable of decoding different Ca^{2+} signals and responding to Ca^{2+} transients in a frequency-dependent manner [60]. Although the physiological significance of this remain largely unexplored, the CaMKII δ_2 isoform might functionally connect to Ca^{2+} sources generated by growth factors receptors through IP_3-mediated Ca^{2+} release and Ca^{2+} entry via TRPC channels. Indeed, the CaMKII δ_2 isoform was shown to be activated in VSMC by growth factors acting on G protein-coupled receptors and receptor tyrosine kinase [46,47]. As argued earlier for other Ca^{2+} signaling molecules, the acquisition of the CaMKII δ_2 isoform by VSMC may be one component of a functional molecular unit that is acquired during phenotypic switching and is necessary to initiate and/or sustain cell proliferation and migration.

What triggers the molecular switch in Ca^{2+} signaling pathways?

Normal VSMC in the vessel wall are quiescent and differentiated but can undergo remarkable and reversible changes into a proliferative phenotype that is likely of fundamental importance during vascular development, regeneration, and normal vascular physiology. It is intuitive to think that it is the maintenance of the proliferative phenotype that contributes to the development of atherosclerotic plaques and neointimal hyperplasia during occlusive vascular disease. Though the molecular mechanisms controlling the phenotypic switch of VSMC have been intensely studied, they remain incompletely understood. The vast majority of studies in the literature have focused on the transcriptional regulation of the contractile genes [70,102]. Most of the regulatory aspects of VSMC contractile genes identified to date have been shown to depend on CArG regulatory sequences found in their promoters and on the binding of the transcription factor serum response factor (SRF) [102,147]. In recent years, increasing number of studies have focused on the ion channels and pumps acquired or lost during VSMC switch to the synthetic phenotype and their role in proliferation [14,32,74,82]. The transcription factors that control the gene regulation of these proteins are starting to emerge [32,75,149,150,160, 162]. However, the pro-proliferative downstream signaling pathways that they regulate remain poorly understood.

What triggers the phenotypic switch? For good reasons, most studies have focused on the identification of growth factors, cytokines, and endothelium-derived factors as initiators of VSMC proliferation [70,102]. The expression levels of TRPC channels appear to be under the control of growth factors. Indeed, studies from one laboratory showed that TRPC6 cation channel was upregulated through the c-Jun/STAT-3 pathway during PDGF-induced pulmonary VSMC proliferation [160], and that TRPC4 was upregulated in ATP-induced mitogenesis through cAMP response element-binding protein (CREB) activation in the same cells [162], providing a mechanism for upregulation of these channels during cell proliferation. Furthermore, SERCA2a expression (or loss thereof) in proliferative VSMC seems to depend on a proliferative signal because expression of SERCA2a can be regained when VSMC are growth arrested [82]. However, the modulation of smooth muscle differentiation markers [54] as well as the reciprocal regulation of the CAMKII isoforms [58] occurs in the absence of VSMC proliferation. In this case, proliferative factors alone cannot account for the change in expression of these proteins and future investigations should consider alternative hypotheses such as loss of endothelial contact/factors, change in matrix interactions, secretion of lipid mediators, mechanical stimuli and oxidative stress and the subsequent signaling pathways they activate. Whether the regulation of the ion channels and pumps during VSMC phenotypic switching can also occur in the absence of serum mitogens under growth arrest conditions remain to be demonstrated. It is tempting to speculate that change in expression of a group of proteins might represent the trigger for the molecular switch in VSMC that occur in a mitogen-independent manner while the maintenance of this switch would require mitogen-induced expression (or repression) of another group of signaling molecules.

The aforementioned idea leads to important questions that remain unexplored: what are the time course and the specific temporal sequence of the molecular changes of Ca^{2+} handling proteins during phenotypic switching of VSMC? What are the transcriptional mechanisms that control change in expression during vascular injury? Insight into the latter question as it pertains to the K^+ channel $K_{Ca}3.1$ expression was gained from a recent study showing that repressor element 1 silencing transcription factor (REST) downregulation is a critical event enabling $K_{Ca}3.1$ expression and proliferation of VSMC [32]. A genome wide analysis using conservative sequences of REST target genes, identified 1892 REST sites in the human genome corresponding to genes transcriptionally repressed by REST and encoding among others: ion channels, growth factors, cytoskeleton proteins, intracellular signaling molecules, and transcription factors [26]. From this study, additional questions arise: What are the ion channels

and pumps whose expression is affected by REST downregulation during VSMC phenotypic switching? What causes REST downregulation in the first place? And what pathway ends this retrogression? Clearly, what happens during VSMC phenotypic switching is likely complex, involves more than one pathway and crosstalk between multiple molecular players that are individually capable of autoregulation through feedback loops.

Ca^{2+} is involved in a wide array of biological functions and the fact that Ca^{2+} regulates the cell cycle is well established. Ca^{2+} has the capability of acting directly on transcription factors (such as DREAM) or indirectly through protein phosphatases (calcineurin/NFAT) or protein kinases (CaMKII or CaMKIV/CREB; PKC/ NF-κB) to induce the activation of numerous target genes. The universality of Ca^{2+} as a second messenger and the ubiquitous requirement for Ca^{2+} signals as activators of many signaling pathways should not be interpreted to mean a lack of specificity. Quite the opposite, Ca^{2+} signals show an impressive variation in their modes of transport, localization, amplitude and frequency and thus their action on downstream transcription factors is remarkably specific. For instance, transcription factors such as NF-κB and NFAT were shown to be activated by different Ca^{2+} signals that vary in their amplitude, frequency and duration; the former is activated by a large and transient Ca^{2+} rise, whereas the latter is activated by low, sustained CA^{2+} signals [39]. The altered expression of Ca^{2+} signaling molecules in proliferating VSMC should instead be interpreted within this paradigm where the source of the Ca^{2+} signal, its amplitude, frequency, and duration are important determinants in VSMC phenotypic switching.

The simplistic view would involve the shift in resting membrane potential to more negative values, the increased expression and subsequent activation (constitutive or agonist-induced) of ROC channels such as TRPC channels that optimally operate at these membrane potential values and the loss of Ca^{2+} pumps responsible for Ca^{2+} clearance would presumably lead to increased basal cytoplasmic Ca^{2+} levels that might be necessary to drive VSMC proliferation. As such, the adaptive changes of VSMC to a non-excitable mode by altering the levels of Ca^{2+} channels, pumps, and Ca^{2+}-activated enzymes appear to be necessary for proliferation. Clearly, future studies on the correlation between the dynamic changes in Ca^{2+} signaling proteins expression during VSMC proliferation and their specific cellular localization in regions of the plasma membrane or in microdomains of the sub-sarcolemmal space are needed, so the precise mechanisms by which these Ca^{2+} signaling molecules promote VSMC proliferation and migration can be fully elucidated.

Conclusions

There is growing evidence that alteration in Ca^{2+} signaling molecules (ion channels, pumps, Ca^{2+}-activated enzymes) expression is closely associated with VSMC phenotypic modulation from a contractile quiescent to a synthetic proliferative phenotype. Figure 1 summarizes the changes in expression of channels and pumps that occur during VSMC phenotypic switching. Here, we argued that these changes in expression are interconnected and each acquired Ca^{2+} signaling molecule represents a component of a pro-proliferative module functioning as a single unit. The functional consequence of these changes is the acquisition by VSMC of a non-excitable-like phenotype, an increased and sustained cytoplasmic Ca^{2+} at more negative membrane potentials and subsequent proliferation. Alternatively, the shift in the source of Ca^{2+} entry from voltage-gated channels to receptor-operated channels would lead to Ca^{2+} entry that connects to pro-proliferative downstream signaling pathways. Interestingly, and regardless of the mechanisms by which this Ca^{2+} signaling switch promotes VSMC proliferation, in most studies where an increased expression of Ca^{2+} signaling molecules have been reported in proliferative VSMC, silencing of these Ca^{2+} signaling molecules have invariably produced an inhibition of proliferation. This highlights the importance of these proteins in VSMC

proliferation and their potential use as targets for novel therapeutic strategies of vascular disease.

The remarkable complexity and variability of Ca^{2+} channels (and ion channels in general) makes them prime targets for drug therapy of disease. For over half a century, pharmacological blockers of the L-type voltage gated Ca^{2+} channels in VSMC have been used in the treatment of cardiovascular diseases, including hypertension and angina [79]. Perhaps the limited therapeutic efficacy of drugs against L-type Ca^{2+} channels is not surprising if some of the vascular pathologies treated with these drugs are characterized by the loss of VSMC L-type Ca^{2+} channel expression, as it is the case during vascular injury. Therefore, it makes implicit sense to focus drug targeting efforts on ion channels that see their expression increased in vascular disease. Among such ion channels, arguably the most attractive current candidates for drug targeting are the TRP superfamily members of channels; this is because of the important role they play in the vasculature and their differential expression among cell types [79,97,136]. While increased TRP channel expression of members of the Melastatin- (TRPM) and Vanilloid (TRPV)-families have been reported in several forms of human cancers ([93, 97] and references therein), their involvement in VSMC proliferation remains unexplored. Nonetheless, in the light of canonical TRPC members increased expression in proliferating VSMC in various disease models, these ion channels could represent realistic targets for therapy of VSMC proliferation in cardiovascular diseases. Similarly, other ion channels ($K_{Ca}3.1$, T-type channels), transporters (NCX) and Ca^{2+} signaling molecules ($CaMKII\delta_2$) that see their expression increased during VSMC proliferation could also represent promising targets for future drug therapy.

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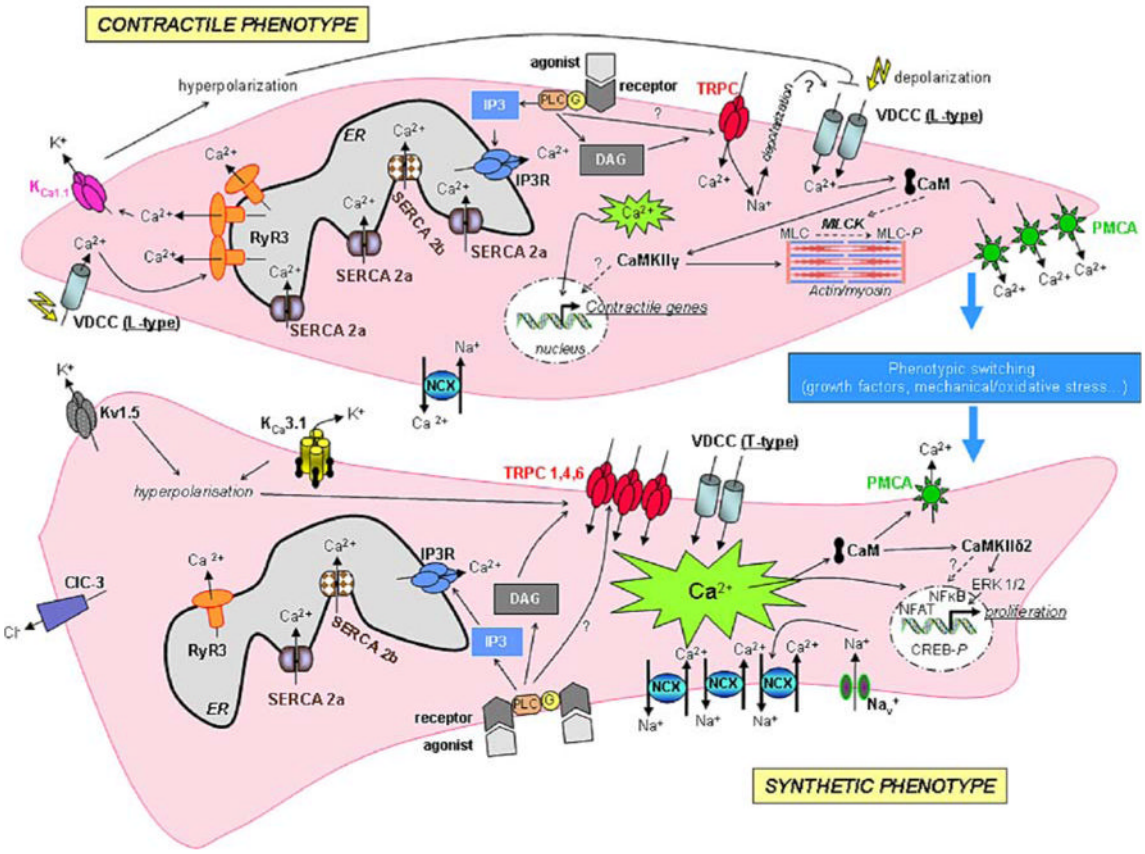

Fig. 1.

Ion channel modulation during phenotypic switching of VSMC. In contractile VSMC, extracellular Ca^{2+} enters the cell mainly through L-type Ca^{2+} channels and Ca^{2+} release occurs through RyR. Vasoactive agonists, by binding to their receptors, mediate Ca^{2+} release from the ER through IP3R and Ca^{2+} entry via TRPC. Intracellular Ca^{2+} maintains precise control of its own homeostasis through regulation of K^+, Cl^-, and Ca^{2+} release channels and Ca^{2+} pumps. Ca^{2+} regulates VSMC contraction through activation of myosin light chain kinase (MLCK) which leads to myosin light chain (MLC) phosphorylation (MLC-P). Vascular tissue injury is associated with a modulation of ion channels, pumps and Ca^{2+}-binding proteins and phenotype modulation to a proliferative synthetic phenotype. Na_v voltage-dependent Na^+ channel; $K_v1.5$ voltage-dependent K^+ channel; ClC_3 voltage dependent Cl^- channel; *VDCC* voltage-dependent Ca^{2+} channel; *CaM* calmodulin