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REVIEW

Excitation-Contraction Coupling and Excitation-Transcription Coupling in Blood Vessels: Their Possible Interactions in Hypertensive Vascular Remodeling

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Summary

Vascular smooth muscle cells (VSMC) display considerable phenotype plasticity which can be studied in vivo on vascular remodeling which occurs during acute or chronic vascular injury. In differentiated cells, which represent contractile phenotype, there are characteristic rapid transient changes of intracellular Ca^{2+} concentration ([Ca2+]i), while the resting cytosolic [Ca^{2+}]i concentration is low. It is mainly caused by two components of the Ca^{2+} signaling pathways: Ca^{2+} entry via L-type voltage-dependent Ca^{2+} channels and dynamic involvement of intracellular stores. Proliferative VSMC phenotype is characterized by long-lasting [Ca^{2+}]i oscillations accompanied by sustained elevation of basal [Ca^{2+}]i. During the switch from contractile to proliferative phenotype there is a general transition from voltage-dependent Ca^{2+} entry to voltage-independent Ca^{2+} entry into the cell. These changes are due to the altered gene expression which is dependent on specific transcription factors activated by various stimuli. It is an open question whether abnormal VSMC phenotype reported in rats with genetic hypertension (such as spontaneously hypertensive rats) might be partially caused by a shift from contractile to proliferative VSMC phenotype.

Key words

Vascular smooth muscle cells • Contractile VSMC phenotype • Proliferative VSMC phenotype • Cell Ca^{2+} handling • Intracellular signaling pathways

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Introduction

Basic function of fully differentiated vascular smooth muscle cells (VSMC) is their contraction for which they express a wide range of contractile and regulatory proteins. VSMC display a considerable plasticity of their phenotype. Under certain conditions such as vascular injury, atherosclerosis or hypertension the fully differentiated cells are able to undergo the process of partial dedifferentiation and to restart the program of cell growth and proliferation. Proliferating VSMC play an important role in the development of blood vessels or in the repair of damaged vessels because they are able to proliferate, to migrate and to synthesize the components of extracellular matrix.

Gary K. Owens has paid a considerable attention to hypertrophy, hyperploidy and hyperplasia of vascular smooth muscle in various forms of experimental hypertension (Owens and Schwartz 1982, Owens and Schwartz 1983). He suggested that VSMC hypertrophy represents an increase of tissue mass that is able to match increased functional demands without the loss of differentiated function. On the contrary, VSMC proliferation is associated with a temporary decrease in expression of smooth muscle-specific contractile proteins (Owens *et al.* 1986, Clowes *et al.* 1989). Thus proliferative VSMC growth may occur under pathological conditions or under the circumstances where functional demands exceed VSMC capacity to respond through cellular hypertrophy (Owens 1989). Numerous growth factors (including angiotensin II, vasopressin and

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endothelin-1), neurotransmitters, mechanical forces and extracellular matrix components (metalloproteinases) can affect the degree of VSMC differentiation and/or induce VSMC growth (with different contribution of hypertrophy and hyperplasia) (Owens 1995, Owens *et al.* 1996). The importance of cooperative interactions between multiple CArG elements (10-bp elements with a sequence $CC(A/T)_6GG$) and serum response factor (SRF) and SRF-accessory proteins (such as myocardin) was summarized by Owens *et al.* (2004) who also reviewed the influence of particular growth factors (angiotensin II, platelet-derived growth factor-BB or transforming growth factor β1) on CArG-mediated mechanisms controlling the expression of smooth muscle-specific differentiation marker genes.

Transition of contractile to proliferative VSMC phenotype is associated with the changes in the expression of ion channels, transporters, receptors and contractile proteins (Matchkov *et al*. 2012). These changes in gene expression lead to the alterations in Ca^{2+} signaling and vascular contractility (Vallot *et al*. 2000). The changes in intracellular cytosolic calcium concentration $[Ca^{2+}]_i$ in differentiated VSMC are large, short-term and locally limited whereas those seen in proliferating VSMC are small, slow and long-lasting. VSMC dedifferentiation is associated with mild elevation of both basal $[Ca^{2+}]_i$ and Ca^{2+} concentration in internal calcium stores (Munoz *et al*. 2013). These two different types (long-lasting and short-term) of Ca^{2+} signaling influence Ca^{2+}-dependent transcription factors and the expression of various genes required for a given state of the cell (Lipskaia and Lompre 2004, Berra-Romani *et al*. 2008).

Aortic VSMC from spontaneously hypertensive rats (SHR) are characterized by a partial shift from a contractile to a proliferative phenotype as indicated by lower levels of contractile-type smooth muscle myosin heavy chain (SM2) and by higher levels of proliferative (synthetic)-type smooth muscle myosin heavy chain (NMHC-B/SMemb) compared to normotensive controls (Umemoto *et al.* 2006). However, the relationship of this VSMC phenotype change to the altered function of vascular smooth muscle in hypertension has not been studied in detail yet.

Vascular remodeling

Vascular remodeling, which occurs during chronic alterations in blood perfusion of vascular bed (Bakker *et al*. 2002) or during reparation of blood vessels following acute vascular injury (Bendeck *et al*. 1994), is an example of modulation of VSMC phenotype. In both cases VSMC phenotype is shifted from a contractile type to a proliferative one.

Vascular remodeling is an adaptive response of blood vessel to various stimuli involving mechanical force which is accompanied by enhanced cytokine secretion (Schober 2008). Progressive changes in vascular wall structure are induced by increased blood pressure (Chen *et al*. 2011) or stent insertion (Chaabane *et al*. 2013). Vascular wall remodeling involves the changes occurring in all three layers (intima, media and adventitia) of vascular wall (Tuttle *et al*. 2001), but our review is focused on the alterations of tunica media, i.e. VSMC.

Remodeling is generally considered to be based upon the reorganization of existing material (Epstein *et al*. 1994). Nevertheless, tunica media remodeling consists of migration, proliferation and apoptosis of VSMC (Buus *et al*. 2001). Several models can be used for the study of remodeling process. Thus, a long-term constriction of isolated arteries (Bakker *et al*. 2000) leads to actin polymerization together with inward remodeling (Staiculescu *et al*. 2013). Small GTPases from Rho GTPase family participate intensively in the remodeling of actin cytoskeleton (Burridge and Wennerberg 2004, Staiculescu *et al*. 2013). Both altered blood flow and long-term vasoconstriction cause vascular wall remodeling which is associated with inflammatory response characterized by macrophage participation and increased expression of pro- and anti-inflammatory cytokines. This inflammatory response enhances remodeling process (Bakker *et al*. 2008).

A moderate increase in shear stress is associated with increased phosphorylation of transcription factor c-Jun (Haas *et al*. 2007) which activates the transcription of matrix metalloproteinases induced by mechanical stress (Ispanovic *et al*. 2006). These metalloproteinases acting on extracellular matrix and on cell surface are also involved in vascular wall remodeling (Sho *et al*. 2002).

Neointimal formation induced by vascular injury and associated calcineurin expression are greater in spontaneously hypertensive rats (SHR) than in normotensive Wistar-Kyoto (WKY) rats (Takeda *et al.* 2008).

Excitation-Contraction Coupling

The stimulation of VSMC by vasoconstrictor agonist leads to the phosphorylation of 20 kDa regulatory

subunit of myosin light chain which increases myosin ATPase activity and enhances vascular contraction (for review see Somlyo and Somlyo 2003). Two counteracting proteins – myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) – participate in the regulation of phosphorylated/dephosphorylated state of the myosin light chain (for the scheme see Fig. 1). Thus, the increased levels of phosphorylated myosin light chain can be achieved by either augmented activation of MLCK *via* calcium-calmodulin action or by the inhibition of MLCP induced through PKC/CPI-17 and/or RhoA/Rho kinase pathways.

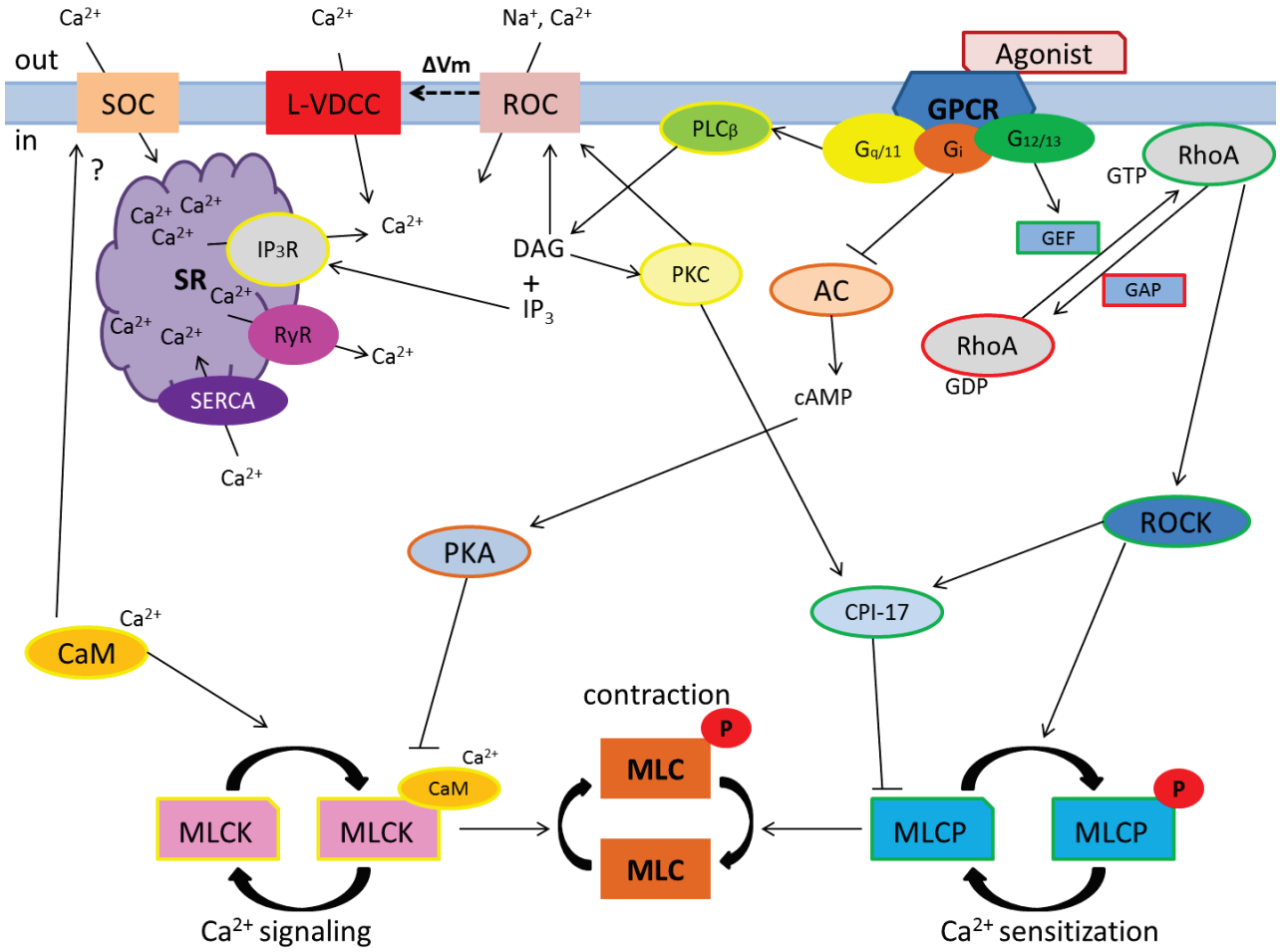

Fig. 1. Excitation-contraction coupling. Vasoconstrictors (such as norepinephrine, endothelin-1, angiotensin II) act *via* receptors coupled with G proteins (GPCR). Receptor stimulation leads to the activation of heterotrimeric G proteins which transmit the signal to the downstream cascade leading ultimately to VSMC contraction. There are three types of α subunits of G proteins – $G_{q/11}$ (yellow oval), G_i (orange oval) and $G_{12/13}$ (green oval). The pathway from $G_{q/11}$ is generally referred as Ca^{2+} signaling while the pathway from $G_{12/13}$ is considered as Ca^{2+} sensitization. $G_{q/11}$ subunit activates phospholipase C_β (PLC_β), which cleaves phosphatidyl inositol-4,5-bisphosphate to diacylglycerol (DAG), and inositol-1,4,5-trisphosphate (IP_3), which binds to inositol trisphosphate receptors (IP_3R) on sarcoplasmic reticulum (SR), causing thus the rise of intracelullar Ca^{2+} concentration $[Ca^{2+}]_i$. Diacylglycerol activates receptor-operated channels (ROC) and protein kinase C (PKC) which might also activate ROC. ROC enable Na^+ entry into the cell, leading thus to plasma membrane depolarization. This depolarization opens voltage-dependent Ca^{2+} channels (L-VDCC). Ca^{2+} is bound to calmodulin (CaM) which then activates myosin light chain kinase (MLCK). Phosphorylation of myosin light chain causes VSMC contraction. Calmodulin also activates store-operated Ca^{2+} channels (SOC) that are responsible for Ca^{2+} refilling of depleted sarcoplasmic reticulum. In the membrane of sarcoplasmic reticulum there are not only IP_3R but also ryanodine receptors (RyR) which are activated by $[Ca^{2+}]_i$ elevation and their activation leads to the depletion of Ca^{2+} stores in sarcoplasmic reticulum, and Ca^{2+} ATPase of sarcoplasmic reticulum (SERCA) which actively transfer Ca^{2+} from cytoplasm into sarcoplasmic reticulum. G_i subunit leads to VSMC contraction by the inhibition of adenylate cyclase (AC). Under the normal conditions cAMP is bound to protein kinase A (PKA) which desensitizes MLCK. $G_{12/13}$ subunit activates guanine nucleotide exchange factor (GEF), which dissociates GDP from G protein RhoA and enables GTP binding on the place, where GDP was bound. RhoA-GTP activates Rho-kinase which phosphorylates and inactivates myosin light chain phosphatase (MLCP). Rho-kinase (ROCK) also phosphorylates CPI-17 and its phosphorylated form binds to the catalytic subunit of MLCP. Further abbreviations: ΔVm – membrane potential change; CPI-17 – smooth muscle-specific inhibitory protein of MLCP; GAP – GTPase-activating protein; RhoA – GTPase of Ras homologue gene family.

Calcium signaling in differentiated VSMC

In contractile cells $[Ca^{2+}]_i$ elevation activates MLCK *via* calcium-calmodulin (CaM) complex (Somlyo *et al*. 1999, Isotani *et al*. 2004). A subsequent phosphorylation of myosin light chain elicits VSMC contraction. Ca^{2+} entry into the cell is generally associated with the activation of $G_{q/11}$ subunit of trimeric G protein. $G_{q/11}$ activates phospholipase C_β (PLC_β) which cleaves phosphatidylinositols into second messengers inositoltrisphosphate (IP_3) and diacylglycerol (DAG) (Wu *et al*. 1992). IP_3 binds to its receptors in sarcoplasmic reticulum and releases Ca^{2+} from internal stores into cytosol (Berridge 1993). DAG activates receptor-operated channels (ROC) which seem to belong to the family of non-selective cationic channels (Albert and Large 2002) (see below). These events lead to membrane depolarization, opening of L type voltage-dependent Ca^{2+} channels (L-VDCC) and massive Ca^{2+} entry into the cell. Ca^{2+} is bound to CaM and this complex activates MLCK (Fig. 1). Ca^{2+} released from sarcoplasmic reticulum opens store-operated channels (SOC) which causes $[Ca^{2+}]_i$ increase through capacitative calcium entry (Smani *et al*. 2004). In parallel, high-conductance, Ca^{2+}-dependent K^+ channels are activated (BK_{Ca}), leading to membrane hyperpolarization and closing of L-VDCC (Nelson *et al*. 1995). $[Ca^{2+}]_i$ level is further modulated by Ca^{2+}-ATPases of sarcoplasmic reticulum or plasma membrane (SERCA and PMCA pumps, respectively) as well as by Na^+/Ca^{2+} exchanger which all lower $[Ca^{2+}]_i$ (Berridge *et al.* 2003, Berra-Romani *et al*. 2008).

Calcium channels in the plasma membrane

In VSMC there are two important types of channels enabling Ca^{2+} entry into the cell. Voltage-dependent and -independent channels transmit different Ca^{2+} signals which have a specific role in the regulation of gene expression and protein synthesis. During the dedifferentiation there is a general transition from voltage-dependent Ca^{2+} entry to voltage-independent Ca^{2+} entry into the cell (Bergdahl *et al*. 2005, Tai *et al*. 2009, Yu *et al*. 2003).

Voltage-dependent Ca^{2+} channels

Voltage-dependent Ca^{2+} channels of L type (L-VDCC) represent a major pathway for Ca^{2+} entry into differentiated VSMC. L-VDCC, which are activated by membrane depolarization, are characterized by a high conductance and a slow inactivation (Catterall 2000). Ca^{2+} entry through L-VDCC is essential for excitation-contraction coupling and thus for VSMC contraction. In addition, L-VDCC can also influence gene expression by the activation of Ca^{2+}-dependent transcription factors (excitation-transcription coupling, see below). The number of functional L-VDCC on the cell surface is decreasing during cell dedifferentiation (Gollasch *et al*. 1998). There are several studies indicating that functional L-VDCC are closely associated with differentiated state of VSMC. The blockade of mitogen-activated protein kinase (MAPK) in proliferating VSMC increased the number of α_{1C} pore-forming subunits of L-VDCC and functional L-VDCC (Ihara *et al*. 2002). MAPK is generally considered to be involved in growth, migration and proliferation of VSMC (Pulver-Kaste *et al*. 2006, Tokunou *et al*. 2001, Xi *et al*. 1999). The *in vivo* blockade of L-VDCC in mesenteric arteries by means of siRNA transfection led to increased basal $[Ca^{2+}]_i$ but reduced vascular contractility and to a decrease in the expression of contractile protein genes in VSMC (Kudryavtseva *et al*. 2014). A further VDCC type – T-VDCC – was found to be expressed preferentially in S phase of cultured aortic VSMC (Kuga *et al*. 1996). T-VDCC are activated at low potential changes, have small conductance and are rapidly inactivated (Perez-Reyes 2003). Both T-VDCC and TRP channels may play an important role in Ca^{2+} entry into dedifferentiated VSMC.

Genetic hypertension in SHR is characterized by sympathetic hyperactivity (Head 1989, de Champlain 1990, Pintérová *et al.* 2011) and by augmented Ca^{2+} influx through L-VDCC (Hermsmeyer 1991, Ohya *et al.* 1998, Cox & Rusch 2002, Sonkusare *et al.* 2006). L-VDCC are upregulated in SHR arteries (Pratt *et al.* 2002) and this upregulation was ascribed to high blood pressure and membrane depolarization (Pesic *et al.* 2004). Nevertheless, it should also be noted that L-VDCC in vascular smooth muscle are activated by norepinephrine (Nelson *et al.* 1988). This is in line with the fact that a great part of sympathetic vasoconstriction, which is augmented in SHR, is susceptible to the inhibition by nifedipine (Paulis *et al.* 2007, Pintérová *et al.* 2010, Zicha *et al.* 2014). Although the above findings support a major importance of sympathetic nervous system for the enhanced participation of L-VDCC in the maintenance of high blood pressure, the alterations in β-adrenergic or NO-dependent vasodilatation might also contribute to the enhanced Ca^{2+} influx in SHR because L-VDCC can be closed by elevated levels of cyclic nucleotides (both cAMP and cGMP) (Ishikawa *et al.* 1993, Orlov *et al.*

1996, Liu *et al.* 1997, Lewis *et al.* 2005, Pintérová *et al.* 2009). Our recent studies revealed enhanced Ca^{2+} entry together with attenuated Ca^{2+} sensitization as characteristic hallmarks of genetic hypertension in SHR (Behuliak *et al.* 2013, 2015).

Non-selective cationic channels

TRP (transient receptor potential) channels in the membrane of VSMC form heterotetrameric cationic channels often permeable for Ca^{2+}. TRP family also involves plasma membrane channels which are activated either by the depletion of internal Ca^{2+} stores (store-operated channels, SOC) or by receptor agonists *via* intracellular ligands (receptor-operated channels, ROC). Among the components of SOC there are proteins of TRPC1 (Bergdahl *et al*. 2005) or other TRP subfamilies (Saleh *et al*. 2006) and probably also pore forming subunit (Orai) and stromal interaction molecule 1 (STIM1) which are involved in the control of SOC activity (Liao *et al*. 2007). Molecular candidates for ROC are mainly TRPC3 and TRPC6 channels (Berra-Romani *et al*. 2008). ROC are opened through a pathway that is independent on internal Ca^{2+} stores. The activation of G protein-coupled receptors (GPCR) by agonists leads to the formation of DAG which stimulates the activation of ROC by a pathway independent of protein kinase C (PKC) (Helliwell and Large 1997) or through its binding to PKC (Albert and Large 2002). The activation of SOC is dependent on internal Ca^{2+} stores and includes the enhanced production of IP_3 which binds to IP_3R and releases Ca^{2+} from sarcoplasmic reticulum. Subsequent $[Ca^{2+}]_i$ elevation stimulates CaM to the activation of SOC (Smani *et al*. 2004). The increased expression of particular TRP channel isoforms and enhanced Ca^{2+} entry through SOC occur during blood vessel cultivation (Bergdahl *et al*. 2005, Yu *et al*. 2003). This might be due to a significant participation of STIM1 and Orai (Potier et al. 2009) which are almost absent in differentiated VSMC (Berra-Romani *et al*. 2008, Potier *et* a*l*. 2009). STIM1 functions as Ca^{2+} sensor of sarcoplasmic reticulum. After the depletion of internal Ca^{2+} stores there is a relocalization of STIM1 in sarcoplasmic reticulum closer to the cytoplasmic membrane (Wu *et al*. 2006). Orai is a pore forming molecule which forms a cationic channel in plasma membrane. Decreased expression of these molecules leads to lower SOC activation (Potier *et al*. 2009). VSMC cultivation increases the expression of both STIM1 and Orai (Berra-Romani *et al*. 2008, Potier *et al*. 2009). During the cultivation there is also a change in SOC activation. Differentiated cells stimulate SOC more through ryanodine receptors (RyR) located on sarcoplasmic reticulum, while dedifferentiated cells lower the contribution of RyR and increase the contribution of IP_3R to the activation of SOC (Berra-Romani *et al*. 2008). This is in agreement with a decreased expression of RyR in proliferating VSMC (Vallot *et al*. 2000). The transition from contractile to proliferative phenotype also substantially increases the activity of ROC (measured as Ba^{2+} entry after administration of permeable DAG analogue) (Berra-Romani *et al*. 2008). Dedifferentiated VSMC show a lower L-VDCC expression (Gollasch *et al*. 1998) so that ROC and SOC mediate a considerable part of Ca^{2+} entry in these cells.

SOC were reported to be exaggerated in preglomerular VSMC of SHR (Fellner and Arendhorst 2002). Capacitative Ca^{2+} entry was also found to be enhanced in SHR but no WKY mesenteric arteries subjected to chronic cyclic stretch (Lindsey and Songu-Mize 2010). The expression of Orai1 and STIM1 is increased in the aorta of stroke-prone SHR in which calcium release-activated calcium channels are highly activated, contributing thus to the abnormal vascular function in this rat strain (Giachini *et al*. 2009). STIM1/Orai1 pathway is more activated in the aorta of male than female stroke-prone SHR (Giachini *et al*. 2012). It should be noted that VSMC derived from the aorta of male SHR migrate and grow faster than those obtained from female SHR (Bačáková and Kuneš 2000).

There are several reports on the alterations of TRPC in arterial smooth muscle of hypertensive rats. The increased expression of TRPC3 channels has been repeatedly reported in SHR (Liu *et al.* 2009, Noorani *et al.* 2011, Adebiyi *et al.* 2012), but some investigators also observed increased expression of TRPC6 and TRPC1 channels in rats with genetic hypertension (Zulian *et al.* 2010, Lin *et al.* 2015). The relationship between TRPC3 or TPRPC6 channels and ROC-mediated calcium entry has been suggested in various forms of experimental hypertension (Liu *et al.* 2009, Pulina *et al.* 2010, Zulian *et al.* 2010). TRPC3 channels are also coupled to IP_3 receptors on sarcoplasmic reticulum in mesenteric VSMC and this coupling is elevated in SHR (Adebiyi *et al.* 2012).

Calcium channels in the membrane of sarcoplasmic reticulum

Inositol trisphosphate receptors (IP_3R) and ryanodine receptors (RyR) are intracelullar Ca^{2+} channels

on the surface of sarcoplasmic reticulum. Both receptor families mediate calcium-induced calcium release (CICR) from sarcoplasmic reticulum which participates in the formation of Ca^{2+} sparks and regenerative Ca^{2+} waves (Berridge 1997). Another important property of IP_3R and RyR is their dual sensitivity to Ca^{2+}. The increase of cytosolic Ca^{2+} concentration initiates the development of positive feedback by opening the neighboring channels, leading thus to a further mobilization of Ca^{2+} from sarcoplasmic reticulum. If $[Ca^{2+}]_i$ is elevated above certain levels, a negative feedback appears which closes these channels (Bootman and Berridge 1996). During VSMC dedifferentiation there is an increase of IP_3R (Berra-Romani *et al*. 2008) which might lead not only to a further $[Ca^{2+}]_i$ elevation but also to the enhanced Ca^{2+}-dependent SOC activation (Smani *et al*. 2004) and further $[Ca^{2+}]_i$ augmentation. IP_3R activation is important for VSMC proliferation (Afroze *et al*. 2007, Wilkerson *et al*. 2006). The induction of proliferation by fetal bovine serum (FBS) or platelet-derived growth factor (PDGF) causes a reduction in the expression of RyR (Vallot *et al*. 2000) which are important for the activation of BK_{Ca} and for closing L-VDCC due to hyperpolarization (Nelson *et al*. 1995). It is possible that lower RyR expression is related to a decreased L-VDCC expression found in dedifferentiated VSMC (Gollasch *et al*. 1998).

Ca^{2+} ATPase of sarcoplasmic reticulum (SERCA)

SERCA is a pump located in the membrane of sarcoplasmic reticulum of VSMC which is powered by ATP and transports Ca^{2+} from cytosol into the lumen of sarcoplasmic reticulum in order to maintain intracelullar Ca^{2+} homeostasis. Two variants of SERCA gene – SERCA2a and SERCA2b – are expressed in VSMC (Lipskaia *et al*. 2014). SERCA2b is characterized by a decreased rate of ATP hydrolysis and Ca^{2+} transport (Lytton *et al*. 1992). Both mRNA and protein of SERCA2a and SERCA2b are present in fully differentiated VSMC. The amount of SERCA2a mRNA rises with age in parallel with the increase of smooth muscle α-actin, which is a marker of VSMC, whereas the amount of SERCA2b mRNA remains constant (Le Jemtel *et al*. 1993). During the dedifferentiation there is a decrease of SERCA2a mRNA (Vallot *et al*. 2000), while the expression of SERCA2b remains unchanged (Lipskaia *et al*. 2005). Other studies reported the increase of SERCA2b expression during VSMC proliferation, whereas the expression of SERCA2a remains unaltered (Berra-Romani *et al*. 2008). In both cases there is a reduction in the ratio between a more efficient SERCA2a and a less efficient SERCA2b. The long-term elevation of $[Ca^{2+}]_i$ is necessary for calcineurin activation and this is prevented by elevated SERCA2a activity (Bobe *et al*. 2011). Calcineurin dephosphorylates further proteins including nuclear factor of activated T-cells (NFAT) which enters into the cell nucleus and induces the expression of genes of proliferative phenotype. In addition, the elevated SERCA2a activity prevents pronounced Ca^{2+} depletion of sarcoplasmic reticulum which is an important component of SOC activation (Bobe *et al*. 2011).

Calcium sensitization

Calcium sensitization, which seems to be independent of Ca^{2+}, is based upon the inhibition of MLCP (Fig. 1). There are two basic components of MLCP inhibition leading to calcium sensitization – phosphorylation of regulatory MLCP subunit (MYPT1) and phosphorylation of smooth muscle-specific MLCP inhibiting protein CPI-17, the phosphorylated form of which is bound to MLCP, leading thus to its inhibition (Eto *et al*. 2004). Rho-kinase (ROCK) is responsible for the phosphorylation of both proteins MYPT1 and CPI-17 (Feng *et al*. 1999, Kureishi *et al*. 1997). CPI-17 is also intensively phosphorylated by protein kinase C (Dimopoulos *et al*. 2007). The inhibitory effect of ROCK on MLCP is counteracted by MLCP activation through cGMP action (Bolz *et al*. 2003). Besides MLCP inhibition ROCK also participates in $[Ca^{2+}]_i$ rise, which is independent of L-VDCC and internal Ca^{2+} stores, but seems to be mediated by ROCK influence on ROC channels activated through extracellular ligands (Ghisdal *et al*. 2003, Kureishi *et al*. 1997). Ca^{2+} signaling in VSMC is usually associated with the activation of $G_{q/11}$ α subunit of heterotrimeric G proteins (Wu *et al*. 1992), whereas the activation of small GTPase RhoA and Ca^{2+} sensitization are associated with the stimulation of $G_{12/13}$ α subunit (Suzuki *et al*. 2003). Some vasoconstrictors (e.g. angiotensin II or endothelin-1) stimulate not only the receptors coupled with $G_{q/11}$ but also the receptors coupled with $G_{12/13}$ (Gohla *et al*. 2000), whereas norepinephrine stimulates α_1- and α_2-adreno-ceptors coupled to $G_{q/11}$ and G_i proteins (Docherty 1998). This suggests a cooperation of Ca^{2+} signaling and Ca^{2+} sensitization. The phosphorylation of myosin light chain is necessary also for the migration and cytokinesis of dedifferentiated VSMC (Fukata *et al*. 2001). During the cultivation of aortic VSMC there is a relative reduction in

the expression of some proteins specific for contractile phenotype (α-actin, myosin heavy chain, h-caldesmon, h1-calponin) while the expression of other proteins is augmented (RhoA, ROCK and MYPT1) (Woodsome *et al*. 2006). ROCK participates in the processes linked to the proliferation of dedifferentiated VSMC (Yamakawa *et al*. 2000).

Our recent data (Behuliak *et al.* 2015) suggest that a decreased expression in CPI-17 might be a cause of reduced Ca^{2+} sensitization in SHR, although enhanced phosphorylation of CPI-17 might partially counteract the functional significance of this alteration in RhoA/Rho kinase pathway.

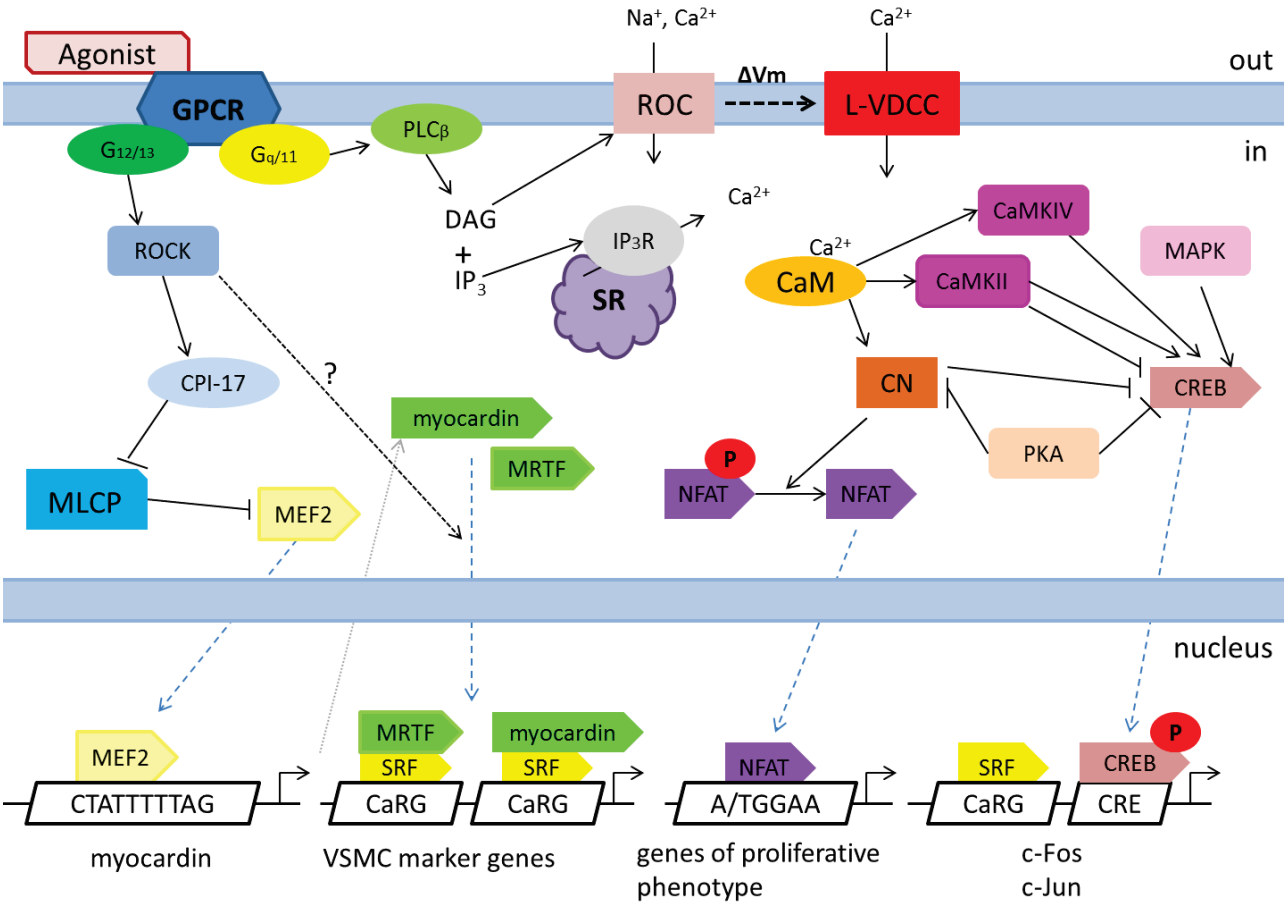

Fig. 2. Excitation-transcription coupling. Vasoconstrictors (such as norepinephrine, endothelin-1, angiotensin II) are acting on receptors coupled with G proteins (GPCR). Stimulation of these receptors leads to the activation heterotrimeric G proteins, which transmit the signal to further components of the respective cascade, leading thus to gene transcription. Rho-kinase (ROCK) phosphorylates CPI-17, which inhibits myosin light chain phosphatase (MLCP), interrupting thus its repressive action on myocyte-specific enhancer factor 2 (MEF2). Transcription factor MEF2 is thereafter translocated to the nucleus, where it activates myocardin transcription. As a consequence of Rho-kinase action myocardin and myocardin-related transcription factors (MRTF) are translocated to the nucleus, where they are bound to serum response factor (SRF) and augment the transcription of genes characteristic for differentiated phenotype. $[Ca^{2+}]_i$ elevation leads to the activation of calmodulin (CaM), which activates calcineurin (CN). CN dephosphorylates transcription factor NFAT (nuclear factor of activated T-cells), that is thereafter translocated into the nucleus to activate the expression of genes of proliferative phenotype. Transcription factor CREB (cAMP responsive element binding protein) might by phosphorylated by various kinases with different effects on CREB activity. Activated CREB is bound on CRE element of gene promoter and together with SRF bound on CaRG element they activate transcription of c-Fos and c-Jun genes. Further abbreviations: CaMK – Ca^{2+}-calmodulin-dependent protein kinase; MAPK – mitogen-activated protein kinase. For other abbreviations see Figure 1.

Excitation-Transcription Coupling

The changes of VSMC phenotype positively correlate with the changes in gene profile expressed by a given cell. Excitation-transcription coupling is a process highly dependent on $[Ca^{2+}]_i$. Specific stimuli may activate various transcription factors depending on extracelullar and intracelullar conditions of the cells. Thus, under various conditions the same agonist might elicit different transcriptional responses; similarly the

different agonists might cause the identical response (divergence and convergence of excitation and transcription coupling) (for details see Fig. 2).

Serum response factor (SRF)

The transcription of most marker genes of differentiated phenotype (smooth muscle α-actin, myosin heavy chain, SM22α, telokin, desmin and h1-calponin) is dependent at least on a single CArG element localized in the gene promoter. CArG element is a 10-bp element with a sequence $CC(A/T)_6GG$ on which SRF is bound (Miano 2003). Basic response of differentiated cells on the stimulation by fetal bovine serum (FBS) is the activation of early genes such as c-Fos. Proximal segment of c-Fos promoter is very sensitive to the stimulation with FBS. This element has the same motif as CArG and also binds SRF protein (Treisman 1992). Thus, SRF stimulates two opposite processes: differentiation and proliferation of VSMC. There are several possible explanations of this paradox. Marker genes of differentiated VSMC usually contain two CArG boxes, whereas c-Fos only one (Spencer and Misra 1996). In some marker genes of VSMC there are C/G substitutions in A/T rich region which lower the binding affinity of SRF (Hendrix *et al.* 2005). An interesting role can be played by myocardin, a highly potent SRF coactivator, the expression of which is increased after the induction of depolarization and Ca^{2+} entry into the cytosol, i.e. the state that corresponds to differentiated VSMC (Wamhoff *et al*. 2004). Myocardin stimulates CArG-dependent expression of marker genes of differentiated phenotype. Myocardin mRNA level decreases during vessel cultivation and the expression of marker genes of differentiated phenotype is reduced in parallel. In contrast, SRF level remains unchanged (Chen *et al*. 2002). On the other hand, after the vascular damage (Hendrix *et al*. 2005) or after PDGF application (Yoshida *et al*. 2007) there is a reduction of myocardin expression with simultaneous decrease in the expression of genes of the differentiated phenotype. Another interesting player seems to be Rho-kinase. Enhanced activation of RhoA stimulates the transcription of smooth muscle α-actin, SM22α and myosin heavy chain (Wamhoff *et al*. 2004). This is probably accomplished by a stimulation of the translocation of myocardin-related transcription factors (MRTF) into the nucleus (Miralles *et al*. 2003). MRTF and myocardin are associated with MADS-box of SRF and they participate in enhancing the target gene transcription (Pipes *et al*. 2006). Myocardin transcription is controlled by transcription factor MEF2 (myocyte-specific enhancer factor 2) (Creemers *et al*. 2006). The expression of MEF2 and marker genes in VSMC is dependent on L-VDCC opening and the activation of ROCK (Ren *et al.* 2010). ROCK phosphorylates CPI-17, which binds to the catalytic subunit of MLCP, ending thus its repressive action on MEF2 protein activity (Pagiatakis *et al*. 2012).

Han *et al.* (2006) reported that SRF is involved on the control of the expression of smooth muscle MLCK (smMLCK) and phosphorylation of myosin light chain (MLC) through MAPK pathway (known also as Ras-Raf-MEK-ERK signal transduction cascade) which is upregulated in SHR (Touyz *et al*. 1999, Kubo *et al.* 2002). The inhibition of either SRF expression or Ras signaling considerably suppressed smMLCK expression in cultured VSMC of normotensive WKY rats (Han *et al.* 2006). SHR are characterized by a 12-base pair insertion adjacent to the CarG box which enhances SRF binding to smMLCK promoter resulting in enhanced smMLCK expression and phosphorylated MLC formation in SHR arteries (Han *et al.* 2006). Chronic *in vivo* inhibition of MEK (tyrosine/threonine kinase), which is increasing SRF occupancy of the smMLCK promoter, has similar blood pressure-lowering effects in SHR as chronic *in vivo* MLCK inhibition (Han *et al.* 2006). The upregulation MAPK/ERK pathway in stroke-prone SHR can be attenuated by chronic antihypertensive treatment by L-VDCC blocker amlodipine or by ACE inhibitor enalapril. Both antihypertensive drugs equally shifted VSMC phenotype towards the differentiated state by reducing nonmuscle myosin heavy chain NMHC/SMemb levels and elevating smooth muscle myosin heavy chain SM2 levels (Umemoto *et al*. 2006).

cAMP responsive element binding protein (CREB)

Transcription factor CREB is considered to be a regulator of VSMC quiescence (Klemm *et al.* 2001). Its aortic content is downregulated in numerous rodent models of hypertension, insulin resistance and diabetes (Watson *et al.* 2001, Schauer *et al.* 2010). CREB downregulation in the above mentioned models of cardiovascular diseases might be related to the action of oxidized LDL through the mechanisms involving reactive oxygen species formation and ERK activation (Schauer *et al.* 2010).

Ca^{2+}-CaM-dependent enzymes are associated with both processes occurring during VSMC contraction or during transcription factor activation. Phosphorylation

of CREB in the promoter is dependent on $[Ca^{2+}]_i$ (Pulver *et al*. 2004). Ca^{2+}-CaM regulates Ca^{2+}-CaM-dependent protein kinase IV (CaMKIV) which activates CREB by its phosphorylation. Phosphorylated CREB is bound to CRE element in the promoter region regulating transcription of genes in both differentiated VSMC (Najwer and Lilly 2005) and proliferating VSMC (Cartin *et al*. 2000). CREB might be phosphorylated by several kinases on different sites and this enables the convergence of multiple signals. CREB is phosphorylated on serine 133 or 142. CaMKIV phosphorylates CREB on serine 133, whereas CaMKII phosphorylates serine 133 and 142. Phosphorylation of serine 142 positively correlates with CREB export from the nucleus (Liu *et al*. 2013) and functions here as a negative regulator of CREB activation. CaMK activity is dependent on $[Ca^{2+}]_i$ and its changes may lead either to the enhancement or to the attenuation of cAMP ability to stimulate transcription depending on the specific CaMK form (Sun *et al*. 1994). Phosphorylation of serine 133 might be mediated by MAPK (Xing *et al*. 1996), cAMP-dependent protein kinase (PKA) (Dash *et al*. 1991) or other kinases. Phosphorylation by MAPK leads to the expression of c-Fos in cerebral arteries (Pulver-Kaste *et al*. 2006) and augments the proliferation and growth of aortic VSMC (Tokunou *et al*. 2001). The whole process can be inhibited by L-VDCC blockers. On the contrary, RyR opening in the membrane of sarcoplasmic reticulum decreases CREB phosphorylation. Ca^{2+} sparks elicited by RyR opening lead to BK_{Ca} activation, membrane hyperpolarization and L-VDCC inactivation (Cartin *et al*. 2000). The application of CaMK inhibitor KN-62 lowers the expression of c-Jun which forms together with c-Fos a complex functioning as a transcription factor of early genes (Pagiatakis *et al*. 2012). The activation of CREB by means of PKA decreases proliferation and migration of pulmonary VSMC (Klemm *et al*. 2001). PKA stimulation leads to a formation of a repressor complex of MEF2 with histone deacetylase 4 and to a decrease in c-Jun expression (Gordon *et al*. 2009). Blood vessels from hypertensive rats have increased basal $[Ca^{2+}]_i$, activated CREB and higher c-Fos expression (Wellman *et al*. 2001). There is no general consensus on the description of signaling pathways leading to gene expression in VSMC with differentiated or proliferative phenotype. It seems that cellular response depends on the particular type of $[Ca^{2+}]_i$ change and the related activation of various intracellular kinases and phosphatases.

Nuclear factor of activated T-cells (NFAT)

NFAT is Ca^{2+}-dependent transcription factor which is permanently expressed in VSMC. Its transcriptionally inactive phosphorylated form, which is present in cytosol, is activated by calcineurin the activation of which is triggered by VSMC excitation and $[Ca^{2+}]_i$ rise. Calcineurin-induced dephosphorylation modifies NFAT conformation and reveals its nuclear localization signal (Okamura *et al*. 2000). Activation of NFAT is negatively regulated by nuclear (Beals *et al.* 1997) and cytosolic kinases (Chow and Davis 2000, Zhu *et al*. 1998). NFAT4 is the main isoform in VSMC. $G_{q/11}$ α subunit of heterotrimeric G proteins activates NFAT4 through $[Ca^{2+}]_i$ rise mediated by IP_3R and L-VDCC. On the contrary, BK_{Ca} activation attenuates the dephosphorylation of cytosolic NFAT4 *via* membrane hyperpolarization, L-VDCC closure and $[Ca^{2+}]_i$ decrease (Stevenson *et al*. 2001). Activated NFAT4 also controls excitability of VSMC through the regulation of gene expression, namely by the decreased expression of voltage-dependent K^+ channel $K_v2.1$ (Amberg *et al*. 2004). The application of α_1-adrenergic agonist phenylephrine into the incubation medium has a chronic positive effect on VSMC proliferation. The activation of NFAT2 by calcineurin following $[Ca^{2+}]_i$ rise participates in this process (Pang and Sun 2009). Permanent SERCA2a activity leads to $[Ca^{2+}]_i$ reduction and to the inhibition of NFAT-induced VSMC proliferation (Lipskaia *et al*. 2005). The activation of NFAT is usually associated with the expression of genes characteristic for proliferative phenotype (Nilsson *et al*. 2007).

Calcineurin expression, which is enhanced during neointimal formation, is augmented in SHR compared to WKY rats (Takeda *et al.* 2008). It should be noted that chronic L-VDCC blockade by continuous nifedipine infusion suppresses the activation of calcineurin in SHR and this is associated with a reduction of cardiac hypertrophy and fibrosis in this hypertensive model (Zou *et al.* 2002). The regression of cardiac hypertrophy in SHR can also be induced by chronic inhibition of Na^+/H^+ exchanger and this regression is accompanied by the inactivation of calcineurin/NFAT pathway (Ennis *et al.* 2007).

VSMC alterations in hypertension

Hypertension is associated with vascular wall remodeling (increased media/lumen ratio) in both human essential hypertension (EH) and genetic hypertension of

the rat, although there is considerable difference between both species in the underlying structural changes. Human EH is characterized by inward remodeling without VSMC hypertrophy and/or proliferation, whereas increased VSMC mass was found in spontaneously hypertensive rats (SHR) (Mulvany 1992). It remains an open question whether these changes observed in the hypertensive subjects/animals are solely the consequence of their high blood pressure or whether they also participate in the pathogenesis of hypertension (Mulvany 1991, Folkow 1995).

Vascular wall hypertrophy (Folkow *et al.* 1973, Mulvany *et al.* 1978) in SHR is caused by VSMC hypertrophy (Owens and Schwartz 1982, Bucher *et al.* 1984) and/or hyperplasia (Head 1991, Devlin *et al.* 1995). Under the *in vitro* conditions VSMC derived from SHR proliferate faster than those originating from normotensive Wistar-Hyoto rats (Yamori *et al.* 1984, Hadrava *et al.* 1991, Saltis and Bobik 1992). Faster *in vitro* proliferation of VSMC and fibroblasts of SHR is associated with augmented responsiveness to various mitogens (Marche *et al.*1995). Although the mechanisms of this important strain difference are still not fully understood, many factors including enhanced expression of transforming growth factor beta (TGF-β) (Hadrava *et al.* 1991, Agrotis *et al.* 1995), hypernoradrenergic innervation (Head 1991) and increased vascular formation of angiotensin II (Mizuno *et al*. 1991, Fukuda *et al.* 1999) or endothelin-1 (Atef and Anand-Srivastava 2014) were proposed to be involved in the above vascular abnormalities of SHR.

There are numerous abnormalities in the contractile function of vascular smooth muscle in the resistance arteries of SHR (for review see Packer 1994, Hughes and Bund 2002, Pintérová *et al*. 2011). These abnormalities, which are responsible for enhanced arterial contraction of genetically hypertensive rats, include greater Ca^{2+} entry through voltage-dependent and/or receptor-operated Ca^{2+} channels (Bruner *et al.* 1986a,b) with a subsequent increase of potassium efflux through Ca^{2+}-activated K^+ channels (Rinaldi and Bohr 1988). Augmented Ca^{2+} entry through L-VDCC (Wilde *et al.* 1994) seems to be a dominant alteration of cell calcium handling in genetic hypertension (Tostes *et al.* 1997) which was confirmed in our studies focused on the role of the contribution of Ca^{2+} entry and Ca^{2+} sensitization to blood pressure maintenance in SHR (Behuliak *et al.* 2013, 2015). However, little attention has been paid to the question whether some functional abnormalities reported in SHR might be related to the augmented fraction of dedifferentiated VSMC in their arteries or not. The same is true for increased passive Ca^{2+} entry into VSMC of non-stimulated SHR arteries (Fitzpatrick and Szentivanyi 1980, Winquist and Bohr 1983). Thus, cell calcium handling in differentiated and dedifferentiated cells or normotensive or hypertensive animals would deserve a careful detailed investigation in the future. This task might be complicated by the fact that cell calcium handling might be different in large (conduit) and small (resistance) arteries (Storm *et al.* 1992).

Conflict of Interest

There is no conflict of interest.

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Abbreviations

ΔVm – membrane potential change; ACE – angiotensin converting enzyme; CaM – calmodulin; CaMK – Ca^{2+}-calmodulin-dependent protein kinase; cAMP – cyclic adenosine monophosphate; CICR – calcium-induced calcium release; CN – calcineurin; CPI-17 – smooth muscle-specific inhibitory protein of MLCP; CREB – cAMP responsive element binding protein; DAG – diacylglycerol; ERK – extracellular signal-regulated kinase; GAP – GTPase-activating protein; GDP – guanosine diphosphate; GEF – guanine nucleotide exchange factor; GPCR – G protein-coupled receptors; GTP – guanosine triphosphate; IP_3 - inositol-1,4, 5-trisphosphate; IP_3R –inositol-1,4,5-trisphosphate receptor; LDL – low-density lipoprotein; L-VDCC – voltage-dependent Ca^{2+} channels; MAPK – mitogen-activated protein kinase; MEF2 – myocyte-specific enhancer factor 2; MEK – tyrosine/threonine kinase; MLC – myosin light chain; MLCK – myosin light chain kinase; MLCP – myosin light chain phosphatase; MRTF – myocardin-related transcription factors; NFAT – nuclear factor of activated T-cells; Orai – pore forming subunit; PDGF – platelet-derived growth factor; PKC – protein kinase C; PLC_β – phospholipase C_β; RhoA – GTPase of Ras homologue gene family; ROC – receptor-operated channels; ROCK – Rho-associated protein kinase; RyR – ryanodine receptors; SERCA – Ca^{2+} ATPase of the sarcoplasmic reticulum; SHR – spontaneously hypertensive rat; SOC – store-operated

channels; SR – sarcoplasmic reticulum; SRF – serum response factor; STIM1 – stromal interaction molecule 1; TGF-β – transforming growth factor β; TRPC – transient receptor potential canonic channels; VSMC – vascular smooth muscle cells; WKY – Wistar-Kyoto rat.

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