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The symphony of vascular contraction: How smooth muscle cells lose harmony to signal increased vascular resistance in hypertension

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Introduction

The principle functions of arterial smooth muscle cells include contraction, relaxation and growth. Calcium signaling mechanisms govern the main functions of arterial smooth muscle and trigger specific responses.[1-3] Cytosolic calcium concentrations and calcium signals are finely tuned by intracellular sources such as the sarcoplasmic reticulum, calcium-binding proteins and plasma membrane calcium permeable channels. In hypertension, these mechanisms are substantially modified, promoting a hypercontractile state and arterial wall remodeling. In this review, we will discuss various elements that are central to intracellular calcium handling and signaling in arterial smooth muscle cells. Emphasis will be given to most recent discoveries of components that link intracellular calcium stores to plasma membrane calcium entry channels. Additionally, we will propose a novel paradigm, suggesting that in hypertension, “alarm signals” generated by chronic innate immune system activation and transduced by pattern recognition receptors modulate calcium signaling mechanisms in arterial smooth muscle, promoting vascular dysfunction. Finally, new research directions in the context of calcium signaling in hypertension will be addressed.

Arterial Smooth Muscle Contractile Mechanism and Calcium Handling

Arterial smooth muscle contraction is regulated by receptor or mechanical activation of the contractile proteins actin and myosin.[4] Changes in the membrane potential can also initiate contraction. The phosphorylation state of the light chain of myosin determines the contractile activity of arterial smooth muscle. Specifically, for contraction to occur, myosin light chain (MLC) kinase must phosphorylate Ser 19 of the 20 kDa regulatory MLC, enabling the interaction between myosin and actin.[5, 6] The cycling of the myosin cross-bridges with actin is promoted by energy released from adenosine triphosphate (ATP) by myosin ATPase activity.[7, 8] In some arteries, MLC is in a phosphorylated state in the absence of any external stimuli (i.e., vascular smooth muscle tone).

An increase in cytosolic calcium concentration is the trigger for vascular contraction.[9] Hypertensive patients and animal models of hypertension exhibit augmented vascular contractile responses.[10-12] A defect in the regulation of calcium and calcium signaling plays a role in hypertension-associated vascular dysfunction. Figure 1 illustrates a timeline of seminal scientific observations on calcium handling in arterial smooth muscle as it relates to hypertension[1-3, 13-35] Abnormal calcium handling in arterial smooth muscle cells may

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involve increased calcium entry, increased calcium storage and/or decreased calcium extrusion. Figure 2 illustrates components of Ca^{2+} signaling in vascular arterial smooth muscle that participate in the contractile process.

a. Calcium-dependent Contraction of Arterial Smooth Muscle

In vivo, the intracellular concentration of calcium is several orders of magnitude lower than that in the extracellular fluid and exhibits dynamic changes throughout the cell due to calcium flux. Strategic spatial positioning of intracellular calcium transporters and targets as well as spatial relationship of ion pumps and channels in the plasma membrane determine calcium flux and the fluctuations in intracellular calcium concentrations.[36] The calcium/calmodulin complex activates MLC to phosphorylate the light chain of myosin. The increase in intracellular calcium concentration in response to specific stimuli occurs in a biphasic mode. In response to receptor-dependent or mechanical stimuli, intracellular calcium concentrations increase. The initial rapid increase in cytosolic calcium is due to calcium release from the sarcoplasmic reticulum, whereas the latter phase of calcium increase in the intracellular space is due to calcium entry from the extracellular space through plasma membrane calcium channels. Ligand-receptor interaction on the plasma membrane stimulates phospholipase C, which specifically catalyzes the formation of 2 second messengers, inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 binds to receptors on the sarcoplasmic reticulum (IP3R) and initiates the release of calcium into the cytosol. This calcium binds to calmodulin, causing conformational changes, which allow the interaction of the calcium/calmodulin complex with the MLC. These events subsequently lead to activation of MLC kinase and phosphorylation of the regulatory MLC. DAG, along with calcium, activates protein kinase C (PKC), which phosphorylates specific target proteins (e.g. contractile proteins, regulatory proteins, channels, pumps, etc.) and has contraction-promoting effects.

b. Calcium-sensitization Mechanisms in Arterial Smooth Muscle

The force of contraction induced by ligand-receptor interaction in arterial smooth muscle is greater than that predicted by the actual calcium concentration in the cells, indicating the existence of calcium-independent mechanisms.[37] In addition to MLC kinase, MLC phosphatase has a regulatory role in MLC phosphorylation.[38-40] Activation of MLC phosphatase (or myosin phosphatase) promotes arterial smooth muscle relaxation via dephosphorylation of light chain of myosin. Myosin phosphatase has three subunits: a 37 kDa catalytic subunit (PP1c), a 20 kDa subunit, and a 110-130 kDa myosin-binding subunit (MYPT1). The binding of PP1c with MYPT1 inhibits the enzymatic activity of myosin phosphatase, allowing the light chain of myosin to remain phosphorylated, and thereby, promoting contraction.[41]

The calcium-sensitizing effect has been ascribed to the activation of the small G protein, RhoA, and its downstream effector Rho kinase.[30] RhoA cycles between an inactive GDP-bound and an active GTP-bound state in response to various stimuli.[42] Three classes of regulatory proteins facilitate activation/inactivation of RhoA: a) GTPase-activating proteins (GAPs) increase the intrinsic GTPase activity of RhoA to facilitate the return of the protein to its inactive state, b) guanine nucleotide dissociation inhibitors (GDIs) sequester the GDP-bound form of RhoA and prevent its binding to the membrane, c) Rho-specific guanine nucleotide exchange factors (RhoGEFs) enable the exchange of nucleotide to activate RhoA-GDP to Rho-GTP (active state).[42] Upon activation, RhoA engages downstream effectors such as the enzyme Rho kinase, which phosphorylates the myosin-binding subunit of myosin phosphatase, inhibiting enzyme activity and promoting phosphorylation of MLC and contraction. Rho kinase is found in 2 isoforms: ROCK1 and ROCK2.[43]

Pharmacological inhibition of Rho kinase reduces blood pressure in experimental models of hypertension and induces relaxation in isolated arterial segments.[30]

c. Calcium Entry Mechanisms

Most calcium mobilization within the arterial smooth muscle cells is modulated by calcium entry channels, such as voltage-operated (VOC) and receptor-operated (ROC) channels, and store-operated calcium entry (SOCE) mechanisms. Other pathways, including purinergic receptors, transient receptor membrane potential (TRP) channels and Na^+/Ca^{2+} exchanger (NCX), are also involved in calcium influx mechanisms but they will not be the focus of the current review.

Voltage-operated calcium channels—The function of VOC is regulated by membrane potential. Membrane hyperpolarization leads to VOC closure, whereas depolarization results in VOC opening and promotes contraction.[44] L-Type VOC channels regulate the majority of agonist-induced calcium entry and depolarize in response to stretch, contributing to agonist-induced vasoconstriction and development of myogenic response and vascular tone, respectively. Increases in intracellular calcium concentration following VOC activation results in stimulation of calcium release from intracellular sources (e.g. sarcoplasmic reticulum) via activation of ryanodine receptors (RyR). This event leads to membrane depolarization, which further activates VOC and promotes calcium influx and constriction. Activation of cGMP-dependent protein kinase and further increases in intracellular calcium concentration act as negative feedback mechanisms that lead to the inhibition of VOC and cease of constriction.

Receptor-operated calcium channels—ROC channels are defined as channels where molecules are separate from the ligand-binding protein, are capable of activating a range of G protein coupled receptors via circulating ligands, and are neither VOC nor store-operated calcium channels (SOC).[45] Following ligand binding, G protein coupled receptors, which are coupled to phospholipase C (PLC), activate ROC channels. This event leads to IP3 and DAG generation, subsequently promoting calcium release from the sarcoplasmic reticulum and PKC-associated activation of MLC kinase.[46, 47] Members of the TRP channel family, including TRPC3, TRPC6 and TRPC7, have been shown to be components of ROC channels.[48, 49]

Store-operated calcium entry—The term store-operated calcium entry (SOCE) describes a cellular mechanism by which depletion of calcium content in the endo-/sarcoplasmic reticulum stimulates calcium influx via activation of plasma membrane calcium channels, replenishing intracellular calcium stores. In this mechanism, the endo-/sarcoplasmic reticulum acts as a capacitor.[50] In non-excitable cells, SOCE is mediated by a highly calcium-sensitive, non-voltage-gated, inwardly rectifying current termed calcium release-activated calcium current (CRAC or I_{CRAC}). Earlier studies demonstrated the existence of a diffusible messenger, calcium-influx factor (CIF). CIF production is restricted in the endoplasmic reticulum and its release is triggered by calcium depletion and/or a drop in intraluminal free calcium concentration. Upon its release from the endoplasmic reticulum, CIF induces displacement of inhibitory calmodulin from a plasma membrane variant of calcium-independent phospholipase A2 (iPLA2b), which transduces the signal to store-operated channels leading to their opening and activation of SOCE. Another pathway that has been shown to contribute to SOCE activation is NCX. When this exchanger is operating in the reverse mode it contributes to intracellular calcium depletion and subsequent activation of SOCE.[51]

Stromal interaction molecule 1 (STIM1) was first identified as a calcium sensor.[52, 53] Currently, two members of the STIM family have been identified and characterized: STIM1 and STIM2. Upon calcium depletion STIM1 and STIM2 translocate towards junctional areas of the endoplasmic reticulum, known as puncta formations, which are in close proximity (10–25 nm) to the plasma membrane.[54] STIM translocation is followed by activation of calcium release-activated calcium channels and SOCE in the plasma membrane. STIM2 plays a role in maintaining basal levels of calcium in the endoplasmic reticulum in the absence of agonist stimulation.[55] In the presence of increasing agonist concentration, SOCE is mediated initially by STIM2 and incrementally by STIM1.[56]

Orai1 is a plasma membrane protein, an essential pore subunit of the CRAC channel[57, 58] and the main means of communication between STIM1 and plasma membrane. The association of STIM1 with Orai1 triggers calcium influx, increases I_{CRAC}, and is enhanced by thapsigargin,[59] an inducer of calcium depletion in endo-/sarcoplasmic reticulum. However, the role of direct conformational coupling between STIM1 and Orai1 in SOCE activation and puncta formation has been challenged by evidence showing that Orai1 is not necessary for the accumulation of STIM1 in puncta as STIM1 accumulates in the absence and presence of Orai1.[60] These recent data suggest the existence of additional intermediate elements. Accordingly, STIM1 and Orai1 interact with TRPC channels and TRPC channels may act as SOC in smooth muscle cells.[61, 62]

In contrast to non-excitable cells, where SOCE is mediated by I_{CRAC} channels, in primary arterial smooth muscle cells, SOCE and agonist-induced contractile responses are mediated by nonselective cation channel. The presence of auxiliary mediators, such as iPLA2b and its lysophospholipid products, is required for signal transduction from STIM1 to plasma membrane SOC channels.[63-65] Activation of nonselective cation SOC channels promotes calcium entry not only by mediating SOCE but also by playing the role of a depolarizing trigger for a secondary activation of VOC. This allows for further increases in calcium influx augmenting vasoconstriction.[66, 67]

The interaction of STIM1 and Orai1 plays a critical role in vascular contraction in various vascular beds and vessel types (i.e., aorta, coronary, and cerebral arteries).[32, 64, 66, 68] RNA silencing of STIM1 and Orai1 reduced phenylephrine- and urotensin II-induced contractions in transfected coronary artery rings, whereas depolarization-induced contractions were not affected by downregulation of either Orai1 or STIM1.[64] Further, thapsigargin-induced aortic contractions were attenuated following *ex vivo* treatment with Orai1 and STIM1 antibodies.[32] Genetic manipulation of STIM1 further supports these data. Smooth muscle targeted STIM1 knock out mice had a 26% reduction in α_1 adrenergic-induced aortic contraction in the absence of any effect on depolarization-induced contractile responses.[32, 69]

Aortae from spontaneous hypertensive stroke-prone rats (SHRSP) exhibited increased isometric force responses during the calcium-loading period on the depletion of intracellular calcium stores.[70] Others have shown that the sarcoplasmic reticulum calcium store is larger in aortae from these animals due to enhanced influx of calcium across the sarcolemma.[71] Activation of CRAC channels was enhanced in aortae from SHRSP compared to normotensive controls and CRAC/Orai1, through STIM1, contributed to augmented aortic contractility of hypertensive rats.[32] We have proposed that sex differences in hypertension might be attributed to differences in STIM1/Orai1-mediated SOCE and intracellular calcium handling mechanisms.[72] Force generation in aortae from genetically hypertensive rats was greater in males compared to females but this difference was abolished in the presence of antibodies against STIM1 and Orai1.[72] Further, expression of these proteins was greater in male compared to female hypertensive rats. These studies were performed mostly in conduit

arteries and the relevance of our findings to resistance vessels in the context of hypertension need to be examined.

Toll-like Receptors, Arterial Smooth Muscle Dysfunction, and Regulation of Calcium Handling in Hypertension: A New Paradigm

Long-term experimental efforts by several investigators, including our laboratory, have shed invaluable insight into the physiological mechanisms that are responsible for the pathogenesis of hypertension. Accordingly, previous studies have demonstrated evidence in support of "renocentric, neurocentric, and vasculocentric" views of the etiology of hypertension. These views need not be mutually exclusive. Most recently, low-grade inflammation and activation of the adaptive arm of the immune system have been implicated in hypertension, offering a potential link among the previously tested hypotheses and a new explanation for the multi-system effects of hypertension.[73, 74]

Recent studies show that host-derived molecules released to the extracellular space due to cell injury and/or death [damage-associated molecular patterns (DAMPs)] can trigger an inflammatory response via activation of the innate immune system. DAMPs include extracellular matrix components, plasma membrane, nuclear, and cytosolic proteins, and elements of damaged/fragmented organelles. Similar to pathogen-associated molecular patterns DAMPs stimulate pattern recognition receptors of the immune system, such as the Toll-like receptors (TLRs), eliciting an inflammatory response.

We recently reported that TLR4 was upregulated in resistance arteries of spontaneous hypertensive rats (SHR) and that TLR4 augmented activation contributed to increased contractile responses to norepinephrine and to elevated blood pressure levels in this rat model of hypertension.[75] The cellular mechanism for these events was related to a COX-dependent mechanism since inhibition of COX-1 and COX-2 reduced contractile responses to norepinephrine in arteries from SHR but not in arteries from SHR treated with anti-TLR4.

Pathogen-associated molecular pattern recognition by TLRs and pathogen clearance after immune complex formation by engagement of Fc receptors are central mechanisms that trigger immune and inflammatory responses.[76-79] In an analogous way necrotic cell death is increased in hypertension giving rise to DAMPs. Various DAMPs are associated with hypertension (e.g. mtDNA, Angiotensin II, oxidized lipoproteins, see ref. #80)[80]. There is cross talk between Fc receptors and TLRs to activate downstream signaling via PLC. Augmented PLC characterizes the accentuated vasoconstrictor response in hypertension, and this may be one mechanism where calcium signaling is amplified in hypertension. We propose that in hypertension, elevated levels of DAMPs activate TLRs, which then signal directly or through cells of the adaptive immune response to elicit an inflammatory process in organs and systems that regulate blood pressure.[80-82]

Future Research Directions: Promising Therapeutic Targets in Hypertension

Fibrocytes

From a traditional viewpoint, resident or adventitial fibroblasts have been thought to be activated by proinflammatory stimuli to proliferate and migrate to sites of vascular injury where they secrete collagen.[83] However, recent work describes an important role for fibrocytes, cells that are implicated in chronic inflammation, fibrosis and wound healing. These cells are derived from bone marrow and are present in atherosclerotic lesions. They have inflammatory features of macrophages and vascular remodeling properties of

fibroblasts. Additionally, fibrocytes develop α-actin expression and a contractile phenotype in tissue culture. Keeley et al[84] observed increased circulating levels of fibrocytes in patients with hypertension and there was a strong correlation between left ventricular mass index and fibrocyte number (total and activated). Circulating fibrocytes are also increased in patients with pulmonary hypertension and are associated with remodeling of pulmonary vessels. In the cochlea, elevation of calcium in fibrocytes surrounding regulatory vessels of the spiral ligament results in propagation of a calcium signal in neighboring vascular cells.[85] Fibrocytes may play an important role in vascular remodeling in systemic hypertension and they may contribute to disturbances in cellular calcium handling in the vascular wall.

Neuropilin

Neuropilins (NRPs) are transmembrane glycoprotein receptors for class III semaphorins and VEGF family members, that together with co-receptor plexins are involved in regulation of angiogenesis and axonal guidance.[86] It was recently revealed that the adult expression of NRP2 is enriched in smooth muscle, where it mediates cytoskeletal rearrangement and a negative regulation of the Rho/ROCK pathway. Thus, NRP2 activation via semaphorin stimulation decreased active RhoA and phosphorylation of MLC in arterial smooth muscle cells and deletion of NRP2 increased contractile responses of bladder smooth muscle.[87] However, the presence and functional importance of NRP2 has not been investigated in the vasculature of hypertensive animals or subjects.

Guanylyl cyclase or nucleotidyl cyclase

Most studies on isolated arteries from hypertensive animals and humans indicate that sodium nitroprusside-induced relaxation does not differ from normotensive values. This has been interpreted to indicate that soluble guanylyl cyclase (sGC) activity is unchanged in hypertension. However, the role of guanylyl cyclase in vasoconstrictor events may need to be re-evaluated. Chan and colleagues observed that hypoxia-induced contraction of porcine coronary arteries was inhibited by inhibition of sGC.[88] Hypoxia also caused contraction in arteries treated with exogenous cyclic inosine 3',5'-monophosphate (cIMP) but not cyclic guanosine monophosphate (cGMP).[89] Using liquid chromatography–mass spectrometry (HPLC-MS), an increased level of cIMP level was measured in arteries exposed to hypoxia. Altitude-induced systemic hypertension is related to hypoxia and it may be that second messengers in vascular arterial smooth muscle generated by a dysregulated sGC contribute to an exaggerated vasoconstriction. A recent study by Beste and co-workers[90] demonstrates that sGC has a broader activity and may more correctly be termed nucleotidyl cyclase. They observed that purified recombinant rat sGC was capable of synthesizing seven cyclic purine and pyrimidine nucleotides. Study of the specificity of nucleotidyl cyclase activity in arteries from hypertensive animals may identify unique contractile signaling molecules related to this enzyme.

Conclusions/Perspectives

Recent evidence from our laboratory supports that in hypertension, elevated levels of DAMPs activate TLRs, which then signal directly or through cells of the adaptive immune response to elicit an inflammatory process in organs and systems that regulate blood pressure. We propose a new paradigm, according to which, the effects of TLR activation on vascular function are transduced via PLC-dependent amplification of calcium signaling, leading to excess vasoconstriction and contributing to vascular resistance. Additional molecular mechanisms presented in this review, involving circulating fibrocytes, neuropilins, and guanylyl cyclase, offer new ways of thinking about the origins of hypertension and hypertensive disorders; however, their effects on fundamental functions of arterial smooth muscle cells and calcium signaling warrant further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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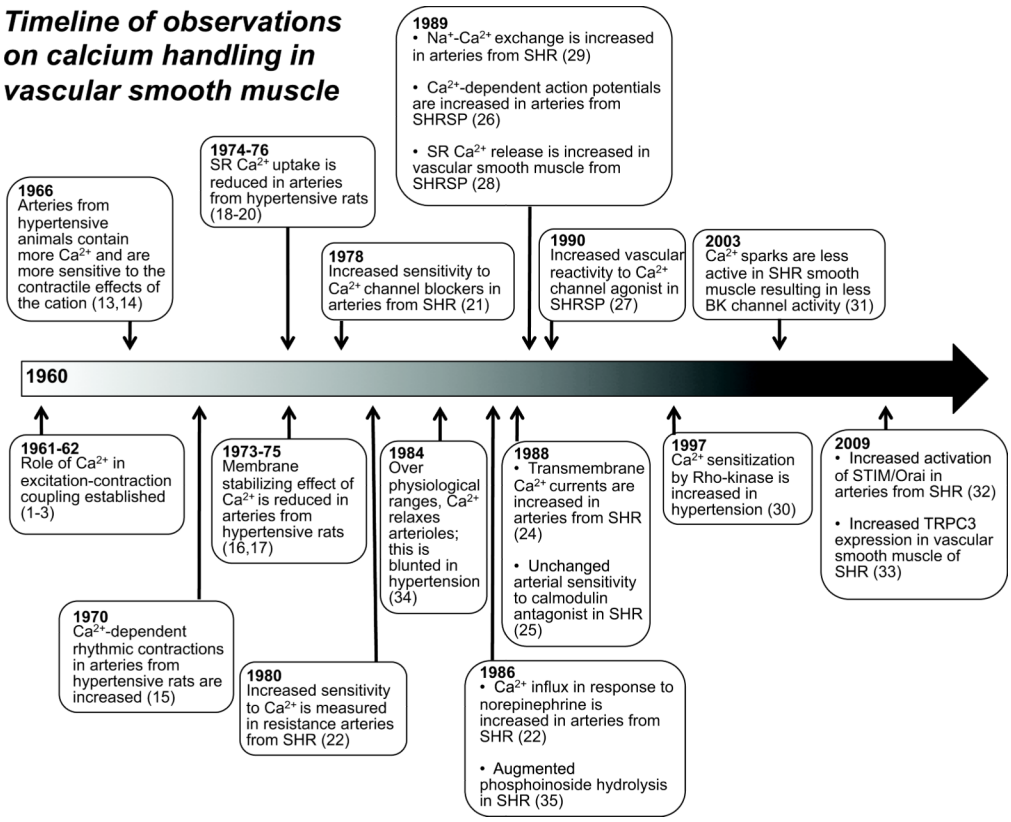

Figure 1.

Timeline of observations on calcium handling in arterial smooth muscle related to hypertension (1960 to 2013). A clear role for calcium (Ca^{2+}) as the activator for contraction in arterial smooth muscle was established in early 1960s. In the mid-60s, observations by several investigators demonstrated that arteries from hypertensive animals are more sensitive to the contractile effects of the cation. Over the following five decades, specifics about signaling cascades regulating intracellular Ca^{2+} in arterial smooth muscle of hypertensive animals and human subjects have been defined in greater detail. References for each observation are parenthetical. Abbreviations: Large conductance Ca^{2+}-activated potassium channel (BK); sarcoplasmic reticulum (SR); spontaneously hypertensive rats (SHR); stroke-prone SHR (SHRSP); stromal interaction molecule (STIM) transient receptor potential cation channel (TPRC).

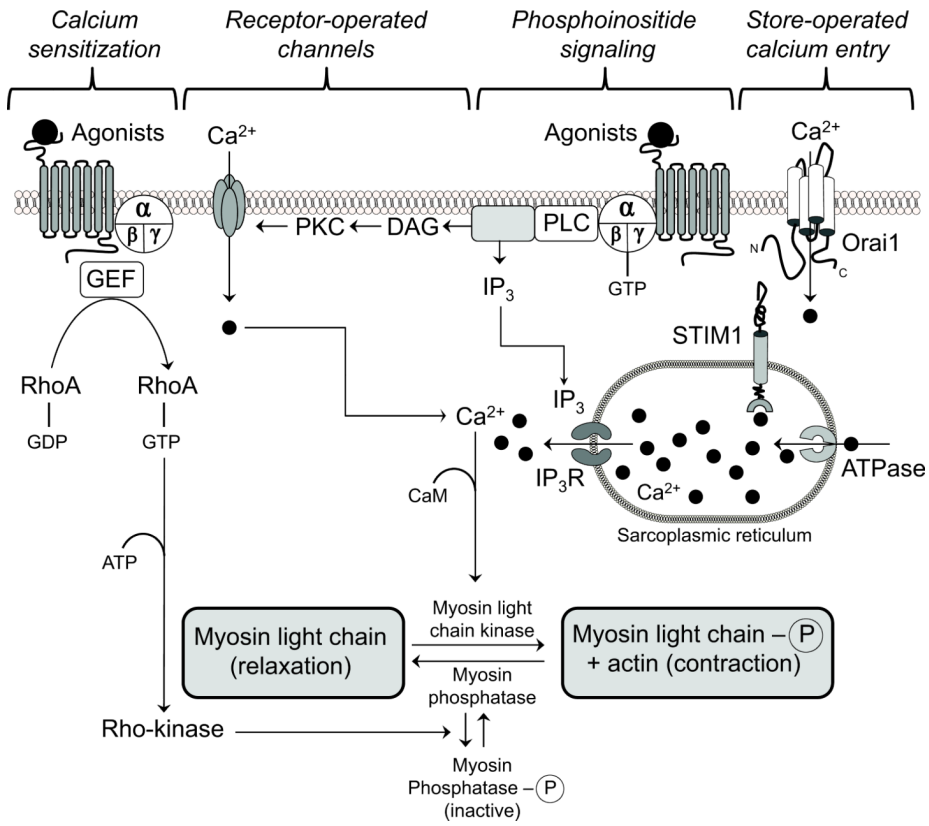

Figure 2.

Components of Ca^{2+} signaling in arterial smooth muscle that participate in the contractile process. The extracellular concentration of free Ca^{2+} is approximately 1.6 mmol/L whereas the intracellular concentration of the cation is 10,000-fold less (50-100 nmol/L). Abbreviations: adenosine triphosphate (ATP); diacylglycerol (DAG); guanine nucleotide exchange factor (GEF); guanosine diphosphate (GDP); guanosine triphosphate (GTP); inositol trisphosphate (IP_3); IP_3 receptor (IP_3R); phosphoinosite phospholipase C (PLC); phosphatidylinositol 4,5-bisphosphate (PIP_2); protein kinase C (PKC); stromal interaction molecule (STIM).

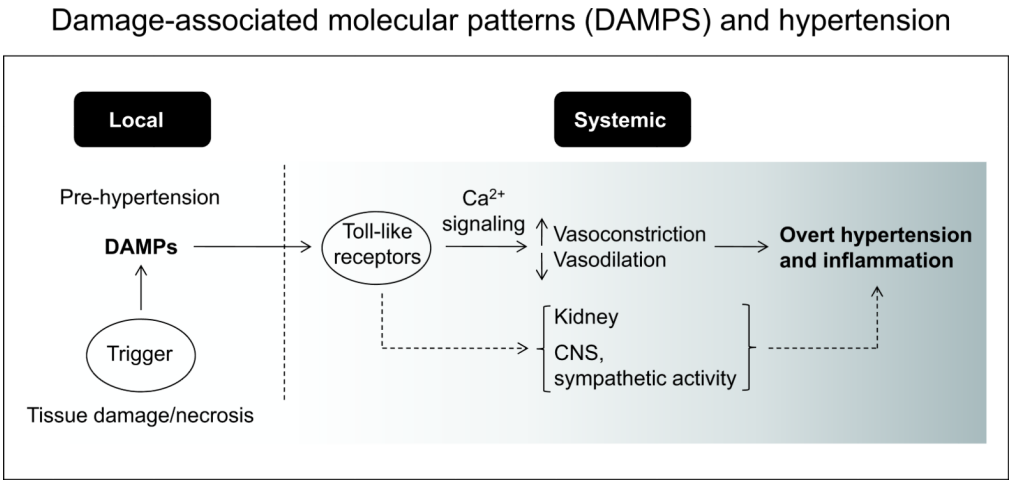

Figure 3.

Damage-associated molecular patterns (DAMPs) and hypertension. We hypothesize that in the pre-hypertensive state, necrotic cell injury during hypoxic and ischemic events leads to the local release of DAMPs. DAMPs activate Toll-like receptors (TLRs) in the arterial smooth muscle cell leading to disturbed Ca^{2+} signaling. This altered Ca^{2+} pattern leads to increased vasoconstriction and reduced vasodilation contributing to overt hypertension. Additionally, activation of TLRs leads to a generalized inflammatory state, characteristic of hypertension. Considerable evidence indicates that activation of TLRs in the kidney and central (CNS) and sympathetic nervous system contribute to hypertension. Thus, activation of the innate immune response provides a common pathway to explain “renocentric, neurocentric, and vasculocentric” views of the etiology of hypertension.