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Vascular Smooth Muscle Cell Signaling Mechanisms for Contraction to Angiotensin II and Endothelin-1

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Abstract

Vasoactive peptides, such as endothelin-1 and angiotensin II are recognized by specific receptor proteins located in the cell membrane of target cells. Following receptor recognition, the specificity of the cellular response is achieved by G-protein coupling of ligand binding to the regulation of intracellular effectors. These intracellular effectors will be the subject of this brief review on contractile activity initiated by endothelin-1 and angiotensin II.

Activation of receptors by endothelin-1 and angiotensin II in smooth muscle cells results in phospholipase C (PLC) activation leading to the generation of the second messengers inositol trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ stimulates intracellular Ca²⁺ release from the sarcoplasmic reticulum and DAG causes protein kinase C (PKC) activation. Additionally, different Ca²⁺ entry channels, such as voltage-operated (VOC), receptor-operated (ROC), and store-operated (SOC) Ca²⁺ channels, as well as Ca²⁺-permeable nonselective cation channels (NSCC), are involved in the elevation of intracellular Ca²⁺ concentration. The elevation in intracellular Ca²⁺ is transient and initiates contractile activity by a Ca²⁺-calmodulin interaction, stimulating myosin light chain (MLC) phosphorylation. When the Ca²⁺ concentration begins to decline, Ca²⁺-sensitization of the contractile proteins is signaled by the RhoA/Rho-kinase pathway to inhibit the dephosphorylation of MLC phosphatase (MLCP) thereby maintaining force generation. Removal of Ca²⁺ from the cytosol and stimulation of MLCP initiates the process of smooth muscle relaxation. In pathological conditions such as hypertension, alterations in these cellular signaling components can lead to an over stimulated state causing maintained vasoconstriction and blood pressure elevation.

Keywords

calcium channel; Rho-kinase; vasoactive peptides; phosphoinositides

In the vasculature the small arteries and arterioles regulate the majority of blood flow resistance in the circulation. Circulating neurotransmitters, hormones, endothelium-derived factors and even shear stress itself plays a role in modulating smooth muscle tone, and consequently lumen diameter (1-3). The final product is the control of blood pressure and organ blood flow.

Arteries are composed of three layers; the tunica adventitia, tunica media and tunica intima. While the outer and inner layers are composed mainly of connective tissue and endothelial

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cells, respectively, the tunica media is composed of smooth muscle cells (3). All smooth muscle cells, regardless of the stimulus, produce force or contraction through cross-bridge cycling between actin and myosin filaments. Although vascular smooth muscle cells are capable of dynamic changes in gene expression to either contractile or differentiation proteins, the contractile phenotype in vascular smooth muscle predominates (4). The contractile response of vascular smooth muscle is the product of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) activation. In smooth muscle, this process is initiated by a calcium (Ca^{2+}) mediated change in the thick (myosin) filaments (2).

In this review, we will discuss the molecular mechanisms by which two common vasoactive peptides, angiotensin II and endothelin-1, produce smooth muscle contraction. Since intracellular Ca^{2+} is fundamental to the contractile process, we will also provide a brief review of the cellular mechanisms that regulate this cation.

Angiotensin II Signaling

Angiotensin II, which is the predominate bioactive peptide in the renin-angiotensin system, promotes the maintenance of systemic pressure through various mechanisms in the cardiovascular and renal systems (5). Although angiotensin II is crucial to salt and water homeostasis, this peptide is also implicated in several cardiovascular conditions, such as hypertension, atherosclerosis and heart failure, and more specifically in vascular smooth muscle contraction; vascular remodeling including the induction of hypertrophy and hyperplasia. Canonical angiotensin II signaling occurs through a membrane bound heterotrimeric G-protein coupled receptor (GPCR). To date, there are two GPCRs known to mediate angiotensin II function, the AT_1 and AT_2 receptors (5,6).

The AT_1 receptor is expressed in a variety of tissues, including the kidney, heart, adrenal gland, brain, lung and adipose; however, the focus will be on AT_1 receptor activation in arterial smooth muscle. The AT_2 receptor is highly expressed in fetal tissues, then declining quickly after birth. In adults, the AT_2 receptor has a varied tissue distribution as well, and is detectable in the kidney and adrenals, pancreas, ovary, brain, heart and vasculature. In vessels, the AT_2 receptor is not localized on the smooth muscle, but is mainly expressed in the adventitia. After injury, levels of this receptor have been observed to increase, which may explain current dogma suggesting that the AT_2 receptor is involved in cellular growth. In contrast to the vasoconstrictive effects of the AT_1 , the AT_2 receptor has been shown to modulate vasodilation (7). Less is known about AT_2 receptor mechanisms than the; however, it is known that it couples to G_i and activates tyrosine and serine/threonine phosphatases. This receptor isoform has also been documented to trigger the kinin-NO-cGMP pathway (8). However, it is through the AT_1 receptor with which the majority of physiological and pathological actions of angiotensin II is mediated, and will be the focus of the signaling in this review.

The angiotensin II signaling cascade via the AT_1 receptor is a consequence of the particular G-protein and/or signaling cascade which is activated: $\text{G}_{q/11}$, G_i , G_{12} , and G_{13} (5,6). The AT_1 receptor signals through three main pathways: classical phospholipase C (PLC) signaling leading to inositol trisphosphate (IP_3) and diacylglycerol (DAG) cleavage, phospholipase D (PLD), also culminating in DAG generation; and phosphatidylcholine, phosphatidic acid (PA) and phospholipase A_2 (PLA_2), which is responsible for arachidonic acid (AA) and prostaglandin (PG) production. Tyrosine phosphorylation, ie, mitogen activated protein kinase (MAPK), which is distinctive for its role in growth factor and cytokine activation, has been associated with increased levels of AT_1 receptor activation. Non-receptor tyrosine kinase activity has also been associated with angiotensin II, including but not limited to: the Src family of kinases, proline-rich tyrosine kinases (Pyks), extracellular signal-regulated kinases (ERKs), paxillin, focal adhesion kinase (FAK), phosphoinositide 3 kinase (PI3K) and an inflammatory

cytokine pathway, janus kinases/signal transducers and activators of transcription (JAK/STAT) (7). Angiotensin II is a pleiotropic signaling molecule, whose varied pathways is complex and specific, but yet also converges to bring forth multiple responses. This review will focus upon the PLC pathway culminating in vascular smooth muscle contraction.

Recent literature suggests that the AT₁ receptor couples to G_q to mediate vasoconstriction, which is the pathway leading to smooth muscle contraction via PLC (9). In addition, angiotensin II was found to elicit a contractile response via activation of G₁₂/G₁₃ G-proteins and the RGS-RhoGEF signaling pathway. Recently, insights into this mechanism were discovered and will be discussed in a later section (10,11). Following G_q/G₁₁ GPCR activation, second messengers are generated, beginning with PLC activation. PLC is a membrane bound enzyme responsible for cleaving membrane lipid phosphoinositide 4, 5- bisphosphate (PIP₂), resulting in the generation of IP₃ and DAG (2,12). IP₃ and DAG regulate two distinct, but parallel pathways leading to increased intracellular Ca²⁺ and culminating in MLC phosphorylation (2,13,14).

IP₃ regulates calcium release into the cytosol from the sarcoplasmic reticulum, mediated via the ryanodine receptor (RyR). The RyR, which has been shown to be localized centrally as well as peripherally on the sarcoplasmic reticulum (SR), is thought to facilitate optimal binding of IP₃ (15,16). Activation of the RyR opens Ca²⁺ channels, causing a rapid increase in Ca²⁺ concentration. After cytosolic Ca²⁺ concentrations peak and then decline, there is a sustained elevation of Ca²⁺ above that of basal levels (2). This sustained increase in Ca²⁺ is due to receptor-operated Ca²⁺ channels on plasma membrane, facilitating increased cytosolic Ca²⁺ levels from the extracellular space (2,14). The EF hand Ca²⁺-binding protein, calmodulin, is the primary target for elevated intracellular Ca²⁺. The Ca²⁺-calmodulin complex is then able to activate MLCK (2). It is also of importance to note that the Ca²⁺-tension relationship changes for stimulation type and during the time of the contraction. In general, studies show that the receptor-mediated stimulations produce a greater tension for a given Ca²⁺ concentration. Moreover, during the sustained phase of contraction, lower levels of Ca²⁺ are needed, which is referred to as Ca²⁺ sensitization. These are two differential points of regulation for vascular smooth muscle contraction (17).

DAG mediates vascular smooth muscle contraction via activation of protein kinase C (PKC). PKC is a serine/threonine kinase known to exist in several isoforms, all which contribute to various physiological activities and are activated differentially. Of the three known vascular smooth muscle isoforms, PKC α and PKC β are dependent upon DAG activation and intracellular Ca²⁺ concentrations, while PKC ϵ is DAG dependent only. PKC modulates vascular smooth muscle contraction by directly phosphorylating MLCK, which subsequently phosphorylates MLC. Furthermore, PKC activation leads to the activation of several other target proteins promoting smooth muscle contraction. PKC phosphorylates, and activates, ERK1/2, Rho-kinase (p160ROCK), and calmodulin-dependent protein kinase II. In addition, intracellular Ca²⁺ elevation was suggested to regulate angiotensin II-induced epidermal growth factor receptor (EGFR) and ERK activation (11). Various ion channels and ion transporters have been demonstrated as downstream targets of PKC. This phenomenon has been illustrated with the use of the specific PKC agonists (i.e. phorbol esters) which induce smooth muscle contraction (2).

All the earlier events described culminating in MLCK activation, resulting in contraction via the phosphorylation of Ser19 of the 20 kDa regulatory protein of MLC. The phosphorylation of MLC allows for the interaction between actin and myosin filaments. Intrinsic ATPase activity of myosin is crucial for this process, as the hydrolysis and subsequent release of ATP results in the cycling of the myosin cross bridges with actin, causing force generation (3, 18-21).

Smooth muscle cells also contain Ca^{2+} -independent mechanisms to regulate contractility. The process of force generation is mediated by MLCK activation, and subsequent actin-myosin cross-bridging, but the process of force maintenance is mediated in a Ca^{2+} -independent manner (2,18,22). MLCP is the contender, regulating the inactivation of MLC by removing the high-energy phosphate group, promoting smooth muscle relaxation. MLCP is composed of two subunits; one is the catalytic 38 kDa subunit of type 1 protein phosphatase (PP1c, δ isoform) and two other non-catalytic subunits (12,17). This mechanism generates force maintenance by means of Ca^{2+} -sensitization of the contractile proteins. This is signaled by the RhoA/Rho kinase pathway which inhibits the dephosphorylation of MLC by MLCP. RhoA is a small, monomeric G-protein, which is regulated by the binding of GTP. This process is facilitated by nucleotide exchange factors, RhoGEFs, which enable the exchange of GDP for GTP, activating RhoA. Upon RhoA activation, the RhoA-GTP complex is able to activate Rho kinase, a serine/threonine kinase. Rho kinase can then phosphorylate the myosin-binding subunit of MLCP, inhibiting it, and thus preventing the dephosphorylation of MLC, causing maintenance of contraction (2,23,24). In addition to the regulatory effects of Rho kinase, CPI-17 is another protein which regulates MYPT phosphorylation status by inhibiting PP1c, thus inactivating MLCP. CPI-17 phosphorylation occurs in a Ca^{2+} -dependent and independent mechanism. Ca^{2+} dependent PKC was suggested to regulate the rapid phosphorylation, whereas phosphorylation by PKC and Rho kinase occurs independently of Ca^{2+} in the later phase. This has been demonstrated with the use of Rho kinase and PKC inhibitors; however, the effects were dependent upon the type of agonist and tissue used. Its activity and expression is a factor in the contractile state of vascular smooth muscle and has also been shown to be of importance in Ca^{2+} sensitization (12,17). Much needs to be explored regarding MLCP regulation, but regardless, Rho kinase and CPI-17 are acknowledged to be of importance in vascular smooth muscle contraction and force maintenance.

Endothelin-1 Signaling

Endothelin-1, which is a 21-amino acid peptide, is known as one of the most potent endogenous vasoconstrictors as described by Yanagisawa, et al. in 1988 (25-27). Since its discovery, the endothelin-1 system has become increasingly complex; several endothelin-1 isoforms and receptors have been identified in a variety of tissues including neuronal, renal and vascular tissues. Interestingly, although some data show a correlation between endothelin-1 levels and hypertension, these data are not conclusive (4). Overall, endothelin-1 signaling in the vasculature is essentially the same as for angiotensin II signaling. A brief overview will be discussed, but the reader is directed to angiotensin II-GPCR coupled signaling pathways for a more in depth explanation. Differing pathways regarding to endothelin-1 signaling itself will be discussed further.

Endothelin-1 is the main isoform secreted by the endothelium, and has been shown to act in a paracrine or autocrine manner on its receptors in vascular smooth muscle. There are three known endothelin-1 receptors with have an assorted tissue distribution and functional role; ET_A and ET_B are present in mammals and a putative ET_C in non-mammals. The typical receptor found on vascular smooth muscle cells is the ET_A receptor, which mediates the vasoconstrictor effects of endothelin-1 (4,25,28). Endothelin-1 receptor activation can lead to diverse responses in the cell through interaction with pathways that are both pertussis toxin-sensitive and insensitive, leading to the conclusion that endothelin-1 acts through several GPCRs. Generally ET_A receptors have been associated with vasoconstriction and cell growth, whilst ET_B receptors are involved in the clearance of endothelin-1, inhibition of endothelial cell apoptosis, the release of NO and PGs leading to vasorelaxation, and inhibition of the expression of endothelin-1 converting enzyme; however, both receptors have been found to elicit vasoconstriction (29-31).

ET_A receptors have been shown to be functionally coupled to the G_{q/11} protein leading to PLC-β activation; G_s linked leading to increased cAMP and also to G_i thus inhibiting adenylate cyclase (30,32-34). Activation of G_{q/11}, ending in IP₃ and DAG cleavage, can stimulate Ca²⁺ release intra- and extra-cellularly, as described for angiotensin II signaling. In addition to second messenger generation, endothelin-1 has also been demonstrated to activate Ca²⁺ channels on the plasma membrane and stimulate Ca²⁺ flux from the extracellular space (4, 35). There is speculation that ET_A receptor activation can activate the Na⁺/H⁺ exchanger, and activate the enzymes PLD generating DAG and PLA₂ releasing AA (4). ET_B receptors in rat carotid were shown to mediate vasorelaxation via a cGMP-NO pathway, the production of vasodilator cyclooxygenase (COX) products and the activation of voltage-activated K⁺ channels. Recently, endothelin-1 was shown to inhibit NADPH oxidase activity and superoxide generation via ET_{B1} receptors (36). The details of these mechanisms are still poorly understood (31).

Both agonists, angiotensin II and endothelin-1, mediate vasoconstriction via an increase in cytosolic Ca²⁺ although mechanisms of this process are different between the two agonists. Endothelin-1 increases intracellular Ca²⁺ by stimulating influx through Ca²⁺ channels and is known to produce a much more sustained contraction by the vascular smooth muscle cells, whereas angiotensin II elicits a potent biphasic response that is generated primarily by mobilization of Ca²⁺ from an intracellular store. Consequently, the actions of angiotensin II actions are relatively rapid in comparison (37-41).

Given the fact that both of these agonists mediate downstream signaling pathways via the same second messengers, it is easy to appreciate how crosstalk occurs between them. Both endothelin-1 and angiotensin II signal through distinct G_q coupled GPCR's leading to phosphoinositide production and MAPK activation. However, the mechanism by which this occurs varies at some points. It has been demonstrated that endothelin-1 and angiotensin II both stimulate this pathway via Ras-Raf; yet, angiotensin II produces phosphorylation of ERK1/2, SAPK/JNK, and p38MAPK via c-Src-dependent pathways. In contrast, endothelin-1 was shown to induce MAPK phosphorylation through c-Src independent pathways (42).

Calcium Mobilization

Given that Ca²⁺ is the trigger for smooth muscle contraction, it is necessary to understand the mechanisms by which intracellular Ca²⁺ levels increase. Hence, this is the focus of much research, and the subject of the remainder of this review. *In vivo*, arterial smooth muscle cells exist with an average intracellular Ca²⁺ concentration which is several orders of magnitude lower than that in the extracellular fluid. Intracellular Ca²⁺ concentration levels do not exist homogeneously throughout the cell, but exhibit dynamic changes in the cell, temporally and spatially, due to Ca²⁺ flux. The changes seen in Ca²⁺ flux are a component of several different events, which are all dependent upon intracellular ultra structure, as well as the spatial relationship of ion pumps and channels in the plasma membrane (4).

Ca²⁺ entry through channels in the plasma membrane and release from the SR are the major sources of intracellular Ca²⁺ (1,4,15). Of the different Ca²⁺ entry channels the voltage-operated Ca²⁺ (VOC), receptor-operated Ca²⁺ (ROC), store-operated Ca²⁺ (SOC) channels and a Ca²⁺-permeable nonselective cation channels (NSCC) modulate most Ca²⁺ mobilization within the cell. We will discuss these particular vascular smooth muscle Ca²⁺ entry channels further below.

Voltage-Operated Ca^{2+} Channels

VOC channels represent a major route by which Ca^{2+} enters vascular smooth cells (43). VOC channel function is regulated by membrane potential such that hyper polarization closes them and depolarization opens them; the latter leading to vasoconstriction (1).

The majority of agonist-induced Ca^{2+} influx probably occurs through L-type VOC channels. Dihydropyridine-sensitive (DHP), L-type VOC channels are regulated by vasoconstrictors that are known to activate the PKC pathway. Vasodilators have also been shown to inactivate these channels; yet through cAMP production (1,44). These gated channels also depolarize in response to membrane stretch, lending support to the hypothesis that these channels are important for the myogenic response and vascular tone. Inhibition of VOCs can occur primarily due to increasing intracellular Ca^{2+} concentration and activation of cGMP-dependent protein kinase (1,2,44).

Endothelin-1 has been shown to activate the VOC channel in smooth muscle cells from porcine coronary arteries, and has also been shown to augment Ca^{2+} channel currents in the smooth muscle cell membrane of guinea pig portal vein. In a model of cultured thoracic aorta vascular smooth muscle cells, Kawanabe and colleagues demonstrated that endothelin-1 produces a sustained increase in intracellular Ca^{2+} levels from VOC channels, as well as others (41,45).

Although endothelin-1 increases intracellular Ca^{2+} by stimulating influx through Ca^{2+} channels, the role of VOC channels is limited, and current data suggest that other channels are more important; namely the NSCC, as evidenced by experiments using nifedipine (41,46). Even though described over sixty years ago, the exact mechanisms of action of this peptide are not completely understood.

Angiotensin II-GPCR stimulation leading to PLC is an accepted mechanism acknowledged to enhance the L-type Ca^{2+} channel. Interestingly, intracellular angiotensin II has been demonstrated to stimulate VOC channels similarly, but yet independently of extracellular ligand binding of angiotensin II to the AT_1 receptor, possibly through the actions of PLC, PKC and/or tyrosine kinase (47,48).

As shown in the smooth muscle cells of the anococcygeous, there are two very distinct mechanisms for inward Ca^{2+} currents and Ca^{2+} store depletion (49). The increase in Ca^{2+} concentration after the activation of VOC channels can also lead to a phenomenon known as Ca^{2+} induced Ca^{2+} release (CICR), where the increase in Ca^{2+} concentration leads to Ca^{2+} release from intracellular stores. The RyR on the SR mediates this process, as they are activated by Ca^{2+} (50). This produces a membrane depolarization that promotes further VOC channel opening and Ca^{2+} entry.

Receptor-Operated Ca^{2+} Channels

Only twenty years ago the exact mechanism of Ca^{2+} channel activation by extracellular ligands, such as hormones and neurotransmitters, was largely unknown. In addition, there was still uncertainty concerning the mechanism by which Ca^{2+} entry was mediated until a model, proposed by Putney and colleagues, detailing the mechanism by which activation of surface membrane receptors causes a sustained Ca^{2+} entry into cells from the extracellular space (51). Furthermore, Benham and Tsien suggested that agonists might activate Ca^{2+} influx directly, by receptor interaction with Ca^{2+} -permeable, NSCC. (52) Currently, ROC channels are defined by the following: channels where molecules are separate from the ligand-binding protein, are capable of activating a range of GPCRs via circulating agonists, and are neither VOC nor SOC channels (28). After ligand binding, ROC channels are generally thought to be activated by GPCRs which are coupled to PLC, leading to IP_3 and DAG generation. (53,54)

Several members of the transient receptor potential channel (TRPC) family are accepted to form ROC channels, including TRPC3, TRPC6 and TRPC7. Angiotensin II has been proposed to activate TRPC6 in rabbit mesenteric arteries, while endothelin-1 activates TRPC3 and TRPC7 (55-57).

Store-Operated Ca^{2+} Channels

SOC channels represent yet another mechanism of excitation-contraction coupling in smooth muscle. SOC channels are activated by means of intracellular Ca^{2+} level depletion, and as a result, are inhibited when Ca^{2+} stores are filled. This process was called capacitative Ca^{2+} entry, before store-operated Ca^{2+} entry (SOCE) was coined. It should be noted that SOCE does not denote any one mechanism of Ca^{2+} entry, nor does it refer to any particular channel (1, 58-62). These channels are highly selective for Ca^{2+} , and have been found to be bradykinin and ATP sensitive (60). RyR or IP_3 sensitive stores can induce Ca^{2+} release sequentially, possibly amplifying the rise in intracellular Ca^{2+} and further stimulating Ca^{2+} channel dependent Cl^- channels. SOC channels were described in 1986, when Putney, *et al.* demonstrated that the same Ca^{2+} entry mechanism normally activated as a result of Ca^{2+} -mobilizing agonists can be triggered equally as well by depleting the intracellular Ca^{2+} pool, even in the absence of receptor activation or elevated cellular levels of inositol polyphosphates (63-65). SOC channel entry has been proposed to increase cytoplasmic Ca^{2+} levels not only for contraction, but also cell proliferation and apoptosis (4). SERCA pumps, which are found on the membrane of the sarcoplasmic reticulum, function by pumping the Ca^{2+} ions back into the SR after a contractile event replenishing Ca^{2+} concentrations (14,15). SERCA pumps can be inhibited by cyclopiazonic acid or thapsigargin, which deplete intracellular Ca^{2+} stores and thereby activate SOC channels (66,67). Ca^{2+} entering through the SOC channels can then be pumped into the stores, replenishing them. According to functional and pharmacological experiments, SOC channel entry elicited by specific inhibitors of SERCA pumps induced a sustained elevation of intracellular Ca^{2+} , which is also dependent upon extracellular Ca^{2+} entry and is attributed to SOCE (68). Thus, Ca^{2+} signals generated in response to receptors involve two coupled components: a transient release of Ca^{2+} stored in the SR, followed by a slowly developing extracellular Ca^{2+} entry (14,15,69,70). With depleted Ca^{2+} stores, Ca^{2+} influx factor (CIF) is generated and diffuses to the plasma membrane where, through a series of reactions CIF activates Ca^{2+} -independent phospholipase A_2 (iPLA $_2$). This generation of lysophospholipids, in turn, activates the SOC channels (62).

The family of TRPC is well known for their role in SOCE, although the mechanism allowing for TRPC activation from store depletion is largely unknown. There is evidence for and against TRPCs actually being a SOC channel, including TRPC1 (56,58,62). Also, interesting data from recent high throughput RNAi screens of thapsigargin-activated Ca^{2+} entry revealed a stromal-interacting molecule (STIM) in *Drosophila* S2 cells and in mammalian HeLa cells, which are now thought to play an essential role in SOCE and conductance through Ca^{2+} -release activated Ca^{2+} (CRAC) channels (71-73). This single membrane spanning protein is now thought to activate the SOC channels by actually sensing Ca^{2+} within the stores. Pharmacological experiments showed that the actual contribution of SOCE to excitation/contraction coupling seems to depend upon the smooth muscle type, and may be more important in tonic smooth muscle (62). A canonical TRP channel, TRPC7, which is activated by AT_1 coupled GPCR activation and DAG, was recently hypothesized to mediate angiotensin II-induced myocardial apoptosis. Moreover, angiotensin II has been postulated to activate TRPC1, as evidenced by data showing that angiotensin II stimulation increased SOCE together with TRPC1 expression. These data were obtained using a cell culture model, but given the heterogeneity of TRPC channels in vascular smooth muscle, there is no reason to believe that this occurrence does not exist *in vivo* (74). In a recent study in vascular smooth muscle cells, endothelin-1 activation of TRPC1 was found to not only be involved in a SOCE, but also in a ROC channel entry (ROCE),

which requires IP₃ receptor activation. A possible explanation for this exciting phenomenon is the report that TRPCs may exist in heterotrimeric complexes, possibly accounting for the varying pharmacological properties seen (56).

Non-Selective Cation Channels

The NSCC, so called because it is 'nonselective', being equally permeable to monovalent cations, such as Na⁺ and K⁺ in the extra- and intracellular compartments (25,28). This channel has been revealed to be activated by stimulation of native ET_A receptors in vascular smooth muscle, and the majority of endothelin-1 response being mediated by NSCCs; namely, NSCC-1 and NSCC-2 (28,41,46,75). It was previously shown, pharmacologically and with electrophysiology, that the response to endothelin-1 is dose-dependent. More specifically, with lower concentrations of endothelin-1, Ca²⁺ entry via IP₃ formation from intracellular stores occurs; however, with higher endothelin-1 concentrations both the former in addition to extracellular Ca²⁺ entry occurs as well (25,28,38,76,77).

The molecular mechanisms of NSCC activation are still not entirely clear. Recent evidence in rabbit internal carotid artery shows that endothelin-1 may induce an intracellular response after GPCR activation via Pyk2 and the ET_A receptor. Data from the same laboratory indicate that PI3K also regulates the activation of Ca²⁺ entry after endothelin-1 GPCR activation and stimulation of NSCC-2 (46,78).

Hot Topics in Vascular Smooth Muscle Signaling

Within just the past year, exciting new insights into vascular smooth muscle signaling have been discovered. We will discuss several recent advancements in the field which have been especially stimulating.

As described earlier, GPCR signaling can elicit stimulatory or inhibitory signals in the cell. With regards to vascular smooth muscle, the G_q/G₁₁ G-proteins have been accepted as producing contraction via the Ca²⁺-dependent PLC pathway; however, Ca²⁺-independent mechanisms, not only for force maintenance, but for contraction have emerged. By means of a novel murine knock-out model for G_q/G₁₁ and G₁₂/G₁₃ G-proteins in smooth muscle cells, Wirth and colleagues elaborated upon the knowledge of how angiotensin II and endothelin-1 mediate contraction via G-proteins. Using aortic segments they demonstrated that in G₁₂/G₁₃ knock-out mice, angiotensin II and endothelin-1 were able to elicit a contraction; however, both potency and efficacy were affected. In the G_q/G₁₁ knock-out mice, although endothelin-1 still produced a severely reduced contraction, angiotensin II was not capable of eliciting any contraction. It was also demonstrated that G₁₂/G₁₃ coupled G-proteins activate the RhoA/Rho kinase signaling pathway via interaction of RhoGEFs; in particular LARG. Using a LARG murine knock-out model, they showed that LARG is necessary for full contraction with angiotensin II and endothelin-1, as demonstrated by the severely restriction constriction elicited in aortic segments from LARG knock-out mice (10,79).

As described earlier, Pyk2 is a Ca²⁺ sensitive non-receptor protein tyrosine kinase that is known to associate with focal adhesion sites and is a downstream effector of angiotensin II and endothelin-1 GPCR stimulation (7,15,80,81). Pyk2 phosphorylation and activation has been postulated in several different pathways, including ERK1/2 signaling in cardiomyocytes, p38MAPK in mesangial cells and recently in RhoA/Rho kinase activation via PDZ-RhoGEF vascular smooth muscle cell migration (15,80,82,83). It has even been suggested that tyrosine kinase pathways such as these may even be important in angiotensin II signaling leading to vasoconstriction (84). These alternative signaling pathways in angiotensin II signaling should be a new directive for investigation into increased vascular smooth muscle reactivity in conditions such as hypertension. This was a focus of a recent paper from Giachini and

colleagues, in press. It is known that increased vascular reactivity to contractile stimuli is present in vessels from DOCA-salt hypertensive mice. Using this model with a pharmacological approach, mechanistic studies were performed to determine the role that Pyk2 plays in the hyper-reactivity exhibited in DOCA-salt hypertension. In mesenteric arteries and aorta, Pyk2 inhibition attenuated the increased vascular constriction to phenylephrine and western blot data showed increased Pyk2 and phospho-Pyk2 in vessels from DOCA-salt treated mice vs. sham, thus confirming a role for Pyk2 in hypertension (80,85).

A recent discovery of a transcription factor named the repressor element 1-silencing transcriptional factor, or REST, was shown to have a consensus sequence, KCNN4 which is known to encode for the intermediate conductance Ca^{2+} -activated K^+ channels (IK_{Ca}). These channels are of the utmost importance in small resistance vessels where a primary vasodilating agent is endothelium-derived hyperpolarizing factor (EDHF). EDHF not only remains elusive as a factor, but the mechanism by which it produces vasodilation is only speculation. One such hypothesis is that K^+ efflux from endothelial cells via these IK_{Ca} channels in coordination with small-conductance Ca^{2+} -activated K^+ channels (SK_{Ca}), activates inward rectifier K^+ channels (K_{IR}). This would lead to vascular smooth muscle relaxation. Unpublished results from the laboratory of R.C. Webb and R.C. Tostes show for the first time that REST expression is closely associated with IK_{Ca} expression in arteries from hypertensive animals. Giachini and colleagues demonstrated that REST expression was down-regulated, whereas IK_{Ca} channels were over expressed in arteries obtained from spontaneously hypertensive stroke-prone rats (SHRSP), when compared to their Wistar-Kyoto controls. Protein levels of SK_{Ca} and IK_{Ca} were also assessed, and alterations were observed in mesenteric arteries from hypertensive animals. It seems as though REST may be a negatively modulatory mechanism, which controls the levels of IK_{Ca} in the SHRSP vasculature.

As mentioned previously, the STIM molecule was shown to play an essential role for SOCE and conductance through CRAC channels (71). The recently discovered subunit of the CRAC channel pore, termed Orai1, is essential for CRAC channel activation (62,72,86,87). However, the exact mechanism for STIM/Orai1 signaling is not yet known. STIM can undergo phosphorylation at specific serine/threonine sites, as well as undergo N-linked glycosylation (88). Sobolff *et al.* determined that while STIM1 is expressed at the cell surface and within the endoplasmic reticulum (ER), the STIM2 protein is expressed only intracellularly, which likely reflects an ER-retention signal that is present in STIM2 but not STIM1 (86,87,89,90). It was apparent that suppressed STIM1 expression, but not STIM2, was able to prevent SOCE and eliminate the store-dependent activation of CRAC channels (71,73). Thus, the function of STIM1 was postulated to act as a Ca^{2+} sensor in the ER (72).

On a final note, the question of 'blame' for hypertension is argued between two schools of thought, cardiovascular and renal physiologists; the former believing that hypertension is due to increased vascular resistance and an overall hyper-reactivity of vascular tissue, the latter believing that the kidney has the final say in blood pressure control. Until recently, renal physiologists everywhere cited papers by Guyton, who was the first to clearly demonstrate the phenomenon of 'pressure natriuresis' and argue for the central role for the kidney in blood pressure control and Coffman, who uses a model of $\text{AT}_{1\text{A}}$ receptor knock-out to illustrate the necessity of the renin-angiotensin system in hypertension (91,92). These elegant studies used the AT_1 receptor knock-out mice and kidney cross-transplantation to show that the $\text{AT}_{1\text{A}}$ receptor is crucial to basal blood pressure regulation, as well as hypertension and that $\text{AT}_{1\text{A}}$ receptors in the kidney are paramount in hypertension and the renal response to hypertension. However, these studies also demonstrated that blood pressure regulation by $\text{AT}_{1\text{A}}$ receptors in non-renal tissues, i.e., vasculature, were also a major contributor to systemic blood pressure (93,94). A paper recently published in PNAS by Michael *et al.*, complements these studies, using a knock-in mutant of the cGMP-dependent protein kinase, $\text{PKG}\text{I}\alpha$. PKGI is expressed

in vascular and smooth muscle cells and has been shown to regulate vascular relaxation via endothelial-derived NO and other nitrovasodilators. The most remarkable data from this study show that the LZM-PKGI α mutant mice exhibit elevated blood pressures, even in the presence of normal renal function and normal renal salt handling, suggesting an important mechanism for vascular smooth muscle in the normal and pathophysiological control of blood pressure (95). Overall, these studies reiterate our awareness of the complexity of hypertension, and the necessity for systemic integration.

Concluding Remarks

Arterial vascular smooth muscle cells constitute the majority of the arterial wall, playing a foremost role in vascular resistance and blood flow. Angiotensin II and endothelin-1 are potent agonists inducing contraction of vascular smooth muscle. The contractile apparatus of vascular smooth muscle, actin and myosin, can be activated in a Ca²⁺-dependent and Ca²⁺-independent manner. Via ligand binding to plasma membrane GPCRs, second messengers are generated and induce the release of Ca²⁺ through channels located on the plasma membrane or on the SR producing a rapid and transient increase in intracellular Ca²⁺. Channels discussed in this review are activated through ligand binding, store depletion and membrane depolarization. In the form of a Ca²⁺-calmodulin complex, subsequent activation of MLCK occurs, inducing contraction via actin-myosin cross bridges. Contraction also occurs, Ca²⁺ independently, through the activation of the RhoA/Rho kinase pathway leading to MLCP inactivation, and the maintenance of contraction.

Given the varied mechanisms for smooth muscle contraction, one can imagine the wide-range of areas where deregulation can occur, leading to increased blood pressure, or hypertension. On a molecular level, alterations in various cellular signaling components, can lead to over stimulation, causing an increased and maintained vasoconstriction, decreased relaxation and consequently, elevation of systemic blood pressure.

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Figure 1. Overview of major contributors to vascular smooth muscle contraction

In smooth muscle, contraction is initiated by a Ca^{2+} mediated change in the thick filaments, or myosin. With myosin light chain phosphorylation, the actin and myosin filaments are capable of interacting. ATP hydrolysis is the source for force generation in smooth muscle; with contraction, inorganic phosphate leaves the myosin head. Relaxation occurs via the action of myosin light chain phosphatase, which de-phosphorylates myosin light chain, inactivating it.

Figure 2. Molecular mechanisms of smooth muscle contraction

Vascular smooth muscle contraction is the summation of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) activity. With receptor binding, an increase in intracellular Ca^{2+} occurs, both via channels located in the membrane and intracellular stores in the sarcoplasmic reticulum. The Ca^{2+} interacts with calmodulin, forming a Ca^{2+} -calmodulin complex which activates MLCK. MLCK can then phosphorylate myosin light chain (MLC-P), allowing for the close interaction of the actin and myosin filaments for force generation. Relaxation occurs with MLCP dephosphorylating MLC. In some instances, force generation can be tonic; this is mediated in a Ca^{2+} -independent manner. Rho-kinase becomes activated via the small, activated RhoA protein, which subsequently phosphorylates MLCP, rendering the enzyme inactive and incapable of de-phosphorylating MLC. In addition, PKC and Rho kinase work in concert to activate CPI-17, which inhibits MLCP. Thus, the vascular smooth muscle cannot relax and a tonic contraction occurs.

Figure 3. The concept of receptor-operated Ca²⁺ (ROC) channels, voltage-operated Ca²⁺ (VOC) channels and store-operated Ca²⁺ (SOC) channels

ROC channels are activated via receptor stimulation of G-protein coupled receptors (GPCRs) directly or through the production of second messengers such as IP₃ or DAG. IP₃ can then bind to and activate the IP₃ receptor on the sarcoplasmic reticulum membrane, causing discharge and release of the stored Ca²⁺. The release of Ca²⁺ from the sarcoplasmic reticulum induces Cl⁻ efflux or the influx of Na⁺ and Ca⁺ from the ROC channels, which can then activate VOC channels. The second messenger, IP₃ activates the IP₃ receptor on the sarcoplasmic reticulum membrane, causing discharge and release of the stored Ca²⁺. After Ca²⁺ depletion from the sarcoplasmic reticulum, the SOC channels are activated. A, agonist; DAG, diacylglycerol; G, G-protein; IP₃, inositol 1,4,5-trisphosphate; IP₃R, IP₃ receptor; PIP₂, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; R, receptor; TK, tyrosine kinase.

Figure 4. New pathways in vascular smooth muscle signaling

Orai1 and STIM1 work together to function as Ca^{2+} sensors within the cell. Orai1 makes up the pore of the CRAC channel, allowing for Ca^{2+} entry while STIM1 senses Ca^{2+} levels within the SR. Their interaction facilitates this process. The IK_{Ca} channels are intermediate conductance Ca^{2+} -activated K^{+} channels which are of importance in small resistance vessels. $\text{G}_{12/13}$ signaling through LARG was recently found to be a mechanism for RhoA/Rho kinase activation, leading to MLCP inactivation.