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Review Article

Ca^{2+} and ion channels in hypoxia-mediated pulmonary hypertension

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Abstract: Alveolar hypoxia, a consequence of many lung diseases, can have adverse effects on the pulmonary vasculature. The changes that occur in the pulmonary circulation with exposure to chronic hypoxia include reductions in the diameter of the pulmonary arteries due to structural remodeling of the vasculature. Although the structural and functional changes that occur in the development of pulmonary hypertension have been well investigated, less is known about the cellular and molecular mechanisms of this process. This review will discuss the role of several potassium and calcium channels in hypoxic pulmonary vasoconstriction, both in elevating calcium influx into pulmonary artery smooth muscle cells (PASMCs). In addition to other signal transduction pathways, Ca^{2+} signaling in PASMCs plays an important role in the development and progression of pulmonary hypertension due to its central roles in vasoconstriction and vascular remodeling. This review will focus on the effect of chronic hypoxia on ion channels and the potential pathogenic role of Ca^{2+} signaling and regulation in the progression of pulmonary hypertension.

Keywords: Intracellular calcium, chronic hypoxia, pulmonary vascular smooth muscle, calcium regulation, hypoxic pulmonary hypertension

Introduction

Sustained pulmonary hypertension is a common complication of chronic lung diseases and alveolar hypoxia is thought to be a key stimulus to the development of this complication. If this disease will not be treated properly, pulmonary hypertension can lead to right-sided heart failure and attendant increases in morbidity and mortality. Exposure to chronic hypoxia (CH) leads to pulmonary hypertension in several animal models: hypoxia leads to structural changes in the walls of distal PA, known as pulmonary vascular remodeling, and a sustained elevation of pulmonary vascular resistance [1, 2]. The characteristic pathological findings in the hypoxic hypertensive pulmonary circulation are increased wall thickness of small muscular arteries and muscularization of normally nonmuscular arteries at the level of the alveolar ducts.

Chronic hypoxic pulmonary hypertension (CHPH) results from the complicated yet poorly understood direct effects of hypoxia and indirect effects of endogenous factors such as endothelin-1 [3-6], angiotensin II [7-10], serotonin [11-13], prostacyclin [14-16], nitric oxide [17-19], platelet derived growth factor [20-22], and metalloproteinases on the cellular and matrix elements of the pulmonary arterial wall. Histologically, progressive hyperplasia and hypertrophy of PASMCs, extension of smooth muscle into previously nonmuscular arteries and other structural changes reduce vascular cross-sectional area, leading to increases in resistances that are not completely reversed by acute administration of vasodilators. The relative contributions of structural remodeling and increased vasomotor tone to CHPH may vary with time, age, species and other factors. The vascular remodeling that occurs in the lung is due, in part, to proliferation and migration of PASMCs [23]. Despite extensive study, the exact mechanisms underlying pulmonary vascular remodeling, growth and migration of PASMCs in pulmonary hypertension remain incompletely understood.

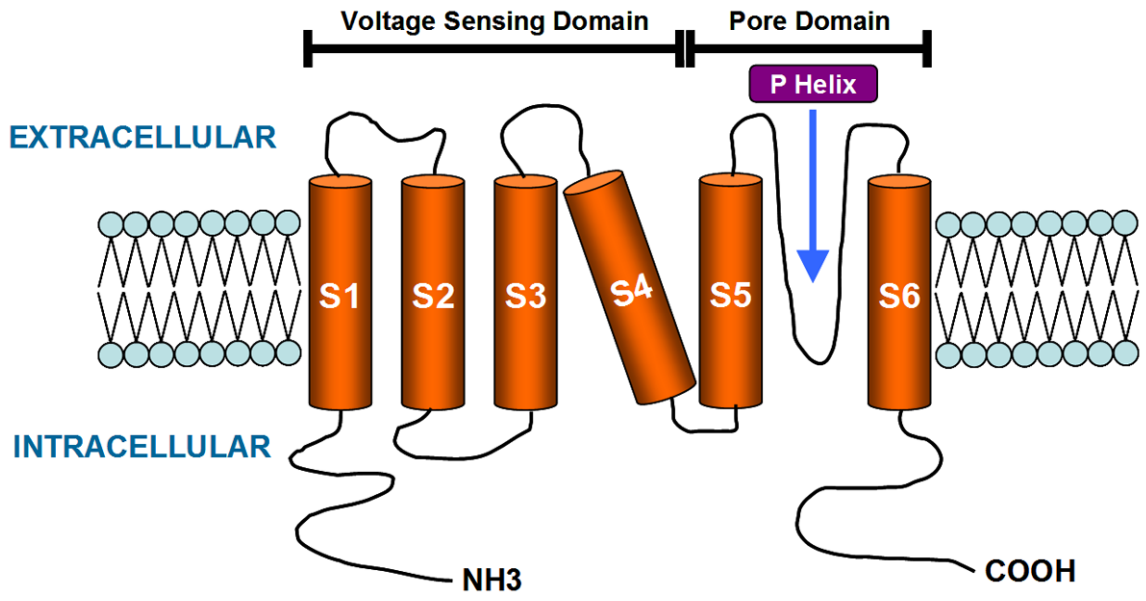

Figure 1. Structure of voltage gated potassium channels.

Ion channels play a very important role in the vascular remodeling that results in chronic hypoxic pulmonary hypertension [24, 25], happened in patients with chronic lung diseases. Many studies have now demonstrated that changes in PASMC function may be related to changes in membrane channel expression and intracellular ion concentrations. Studies on intracellular Ca^{2+}, ion channels, transmembrane ion influx and membrane potential become more and more popular, which provide us more insight and greatly benefit our understanding towards CHPH. An increase in cytoplasmic Ca^{2+} concentration is used as a key signaling messenger for regulating a host of kinetically distinct processes leading to cell growth and proliferation. This leads to inhibition of apoptosis and an increase in cellular proliferation. A better understanding of the pathophysiology of hypoxic pulmonary vasoconstriction and vascular remodeling will enable the design of better treatments for hypoxic and other forms of pulmonary hypertension. In this review, we will focus on CH-induced changes in channel activity in PASMCs and evidence for alterations in channel expression.

Resting membrane potential by special K^+ channels

The membrane potential or better, membrane voltage, is the membrane voltage usually describes the voltage across the plasma membrane between inside and outside of a cell. When the membrane voltage of a cell does not change in time, it is called resting potential (or resting voltage), as opposed to the dynamic potential. The resting potential is mostly determined by the concentrations of the ions in the fluids on both sides of the cell membrane and the ion transport proteins that are in the cell membrane.

For most animal cells, potassium ions (K^+) are the most important for the resting potential. Due to the active transport of potassium ions, the concentration of potassium is higher inside cells than outside. Most cells have potassium-selective ion channel proteins that remain open all the time. There will be net movement of positively-charged potassium ions through these potassium channels with a resulting accumulation of excess negative charge inside of the cell. The outward movement of positively-charged potassium ions is due to random molecular motion (diffusion) and continues until enough excess negative charge accumulates inside the cell to form a membrane potential which can balance the difference in concentration of potassium between inside and outside the cell. “Balance” means that the electrical force (potential) that results from the build-up of ionic charge, and which impedes outward diffusion, increases until it is equal in magni-

tude but opposite in direction to the tendency for outward diffusive movement of potassium. This balance point is an equilibrium potential as the net transmembrane flux (or current) of K^+ is zero.

Structure of voltage gated potassium channels

Grunnet et al found that although some mammalian channels can function as homotetramers or heterotetramers, nearly all voltage gated potassium channels are homotetramers [26]. Every subunit contains six transmembrane a-helices named S1 to S6 and a short hydrophilic helix between S5 and S6 called the P helix. Helices S5 and S6 along with the P helix from each subunit assemble with fourfold symmetry to form the pore domain. The pore domain is a conserved feature in all potassium channels which contain the potassium conduction pathway and gating regions. The S1 to S4 helices of each subunit form the four independent voltage sensing domains controlling the open or close of the channel. Some highly conserved arginine residues are within the S4 helix of Kv channels. These residues give positive charges in every three amino acids within the helix. The number of arginine residues in the S4 helix ranges from three to five depending on the channel [27] (**Figure 1**). This conservation is not only found in Kv channels, but also found in calcium channels, proton channels, sodium channels and voltage dependent phophatases [28]. The Kv channels in eukaryotic cells also contain an N terminal cytoplasmic tetramerisation domain, but this domain is not found in any bacterial channel [29]. Many researchers find that pore domains in Kv have almost the same structure [30-32]. However, people still do not know the structures of the voltage sensing domains of Kv channels.

Changes in K^+ channels with chronic hypoxia

It has been stated in previous sections that membrane potassium channels play an essential role in smooth muscle excitability. In vascular smooth muscle cells (VSMCs), K_V channels are integral in the regulation of membrane potential and vascular tone, therefore inhibition or closure of vascular smooth muscle cell K^+ channels, which are open at the resting membrane potential, causes membrane depolarization. This change in membrane potential activates voltage-gated Ca^{2+} channels, leading to an increase in intracellular Ca^{2+} concentration and vasoconstriction. VSMCs have a high input resistance; therefore, even a small change in K^+ channel activity can have a significant effect on membrane potential and, consequently, vascular tone.

Indeed, in isolated PASMCs, acute hypoxia has been shown to significantly depolarize the membrane potential by about 15-20 mV [33], leading to contraction of individual PASMCs. It is assumed that acute hypoxia acts first to depolarize the membrane by inhibiting the K^+ channels involved in setting the resting membrane potential. The membrane depolarization will then activate voltage-dependent calcium channels and calcium influx, which will lead to increased intracellular Ca^{2+} concentration and vasoconstriction. It has been confirmed that the hypoxia-induced increase in intracellular Ca^{2+} was inhibited by L-type Ca^{2+} channel blockers [34-36] and, that hypoxia-induced constriction of small pulmonary arteries (<300 μm) associated with membrane depolarization could be inhibited by verapamil, a voltage dependent Ca^{2+} channel antagonists [37, 38]. These studies clearly illustrate the importance of Ca^{2+} influx through membrane voltage dependent Ca^{2+} channels. However, because these VDCCs are generally closed at the resting membrane potential of PASMCs, it is likely that hypoxia first act on inhibition of K^+ channels and membrane depolarization.

K^+ channels are the major regulators of resting membrane potential in PASMCs [34, 39], which regulates intracellular Ca^{2+} concentration due to the voltage dependence of Ca^{2+} influx through sarcolemmal Ca^{2+} channels. The change of intracellular Ca^{2+} concentration is required for both HPV [40-42] and smooth muscle growth and proliferation [43-45]. Under normal conditions, voltage-gated K^+ (Kv) channels are the main subtype responsible for control of basal resting membrane potential. The inhibition of K^+ channels caused membrane depolarization, activation of VDCCs and increased intracellular Ca^{2+} concentration [34, 39]. Many people found that depolarization and reduced Kv channel activity in PASMCs from rats exposed to CH. Suzuki H et al found that depolarization in rat main pulmonary artery and small pulmonary artery during chronic hypoxia [46]. Smirnov SV also found that chronic hypoxia was associated

with a marked reduction in amplitude of K^+ current. The resting potential of the PASMCs from chronically hypoxic animals was significantly more positive than that of cells from normoxic animals [47]. These data demonstrated that hypoxia caused alterations in K^+ channel regulation or expression. In vitro experiments showed decrease in Kv channel activity was mediated by transcriptional regulation. We also showed that the mRNA levels of Kv channel alpha subunits, Kv1.2 and Kv1.5 were decreased in prolonged hypoxia (24-60 h) and the protein levels of Kv1.2 and Kv1.5 were also decreased by hypoxia (48-72 h), suggesting that hypoxia could inhibit K^+ channel expression [48].

However, since the effect of in vitro experiments in cultures cells may not reflect the effects of CH on K^+ channel expression in the animal, many people explored the effect of CH on Kv channel expression in vivo. Pozeg ZI et al demonstrated that the expression of Kv1.5 was decreased in adult male Spregue-Dawley rats exposed to CH for 3 to 4 weeks compared with the control [49]. Hong Z et al found that the expression of mRNA for Kv1.2, Kv1.5 and Kv2.1 is reduced in PASMCs isolated from rats kept at 0.67 atmospheres for less than 24 h. These experiments demonstrated that Kv channels may be involved in the signaling of chronic hypoxic pulmonary hypertension [50].

After that, other labs found the mRNA expression of Kv1.1, Kv1.5, Kv2.1, Kv4.3 and Kv9.3 α-subunits decreased in cultured rat PASMCs under chronic hypoxia [39, 51-55]. The response of downregulation of Kv channels is specific to PASMCs [39, 51, 52] and therefore selective for the pulmonary circulation since, until now, chronic hypoxia inhibition of the expression of Kv channels α- or β-subunits have not been reported in mesenteric arterial SMCs. Two animal models including the chronically hypoxic animal model and the Kv1.5 knockout mouse model have been set up and studies based on these two models have emphasized the importance of Kv channels in the pulmonary vascular response. Investigations of freshly isolated PASMCs from chronically hypoxic animal models show downregulation of Kv1.2 and Kv1.5 [36, 56]. Studies with Kv1.5 knockout mice models show impaired hypoxic pulmonary vasoconstriction and reduced O_2-sensitive K^+ current in PASMC. All these research provide strong evidence for the role of Kv channels in the chronic pulmonary vascular response to hypoxia [36, 39, 51-56].

The mechanisms by which the expression of Kv channels was downregulated have been under investigation. Many hypotheses have been put forward to explain the CH-induced inhibition of Kv channels expression in PASMCs, including: 1) upregulation or downregulation of the transcription factors and signal transduction proteins that can directly bind to Kv channel gene promoters and regulate the Kv channel gene transcription [57-59]. 2) Induction of transcription factors that upregulate intermediate inhibitors of the Kv channel genes, such as endothelin-1 [57, 60]. A variety of transcription factors and signal transduction signaling proteins such as HIF-1, nuclear factor-κB, c-fos/c-jun, BMP, P53, KBF, FixL, and FixJ can be modulated by hypoxia [39, 57, 59-71], suggesting that a large number of transcriptional pathways contribute to the response under chronic hypoxia. For example, the ability of HIF-1 to repress Kv channels was demonstrated by the finding that overexpression of HIF-1 under normoxic conditions, using AdCA5, an adenovirus that encodes a constitutively active form of HIF-1α [72], can downregulate the expression of Kv1.5 and Kv2.1 [57]. Although HIF-1 has been shown to regulate the transcription of many genes, the possibility is that HIF-1 could repress transcription of genes encoding Kv channels.

Ca^{2+} is required for pulmonary vasoconstriction

Myosin-light-chain kinase (MLCK) is a serine/threonine-specific protein kinase that phosphorylates the regulatory light chain of myosin II. Three different MLCK isoforms exist. There is a cardiac-MLCK encoded by mylk3, a skeletal-MLCK encoded by mylk2, and smooth muscle-MLCK encoded by mylk. Smooth muscle and non-muscle MLCK are identical and is the product of the same gene, mylk. This protein is important in the mechanism of contraction in smooth muscle. Once there is an influx of calcium into the smooth muscle, either from the sarcoplasmic reticulum or, more important, from the extracellular space, contraction of smooth muscle fibers may begin. First, the calcium will bind to calmodulin (CaM). This binding will activate MLCK, which will go on to phosphorylate the myosin light chain at serine resi-

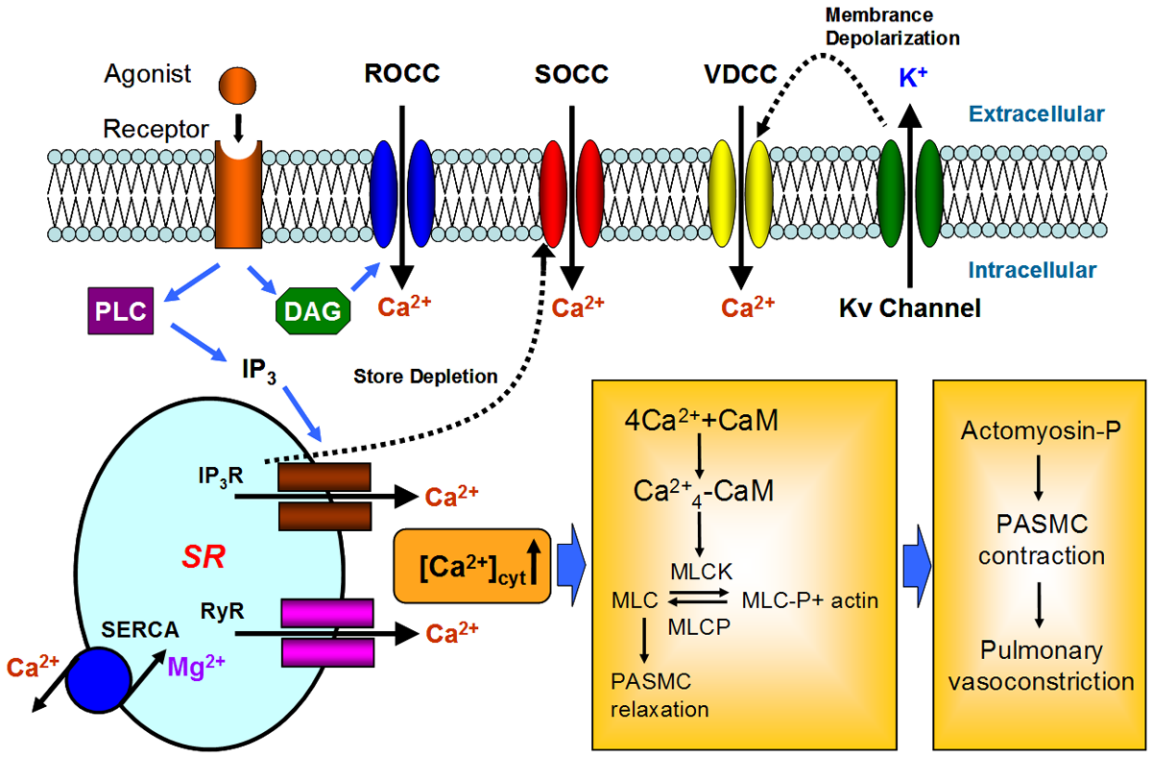

Figure 2. Schematic illustrating increased levels of $[Ca^{2+}]_{cyt}$ in PASMC are required for pulmonary vasoconstriction.

due [73]. This will enable the myosin cross bridge to bind to the actin filament and allow contraction to begin (through cross bridge cycling) (**Figure 2**). Since smooth muscle does not contain a troponin complex like does striated muscle, this mechanism is the main pathway for regulating smooth muscle contraction.

CaM is a ubiquitous Ca^{2+} sensor protein through which a variety of the second messenger effects are mediated. CaM is a 17 kDa Ca^{2+}-binding molecule that has been highly conserved throughout biological evolution. It is composed of an N- and C-terminal lobe tethered by a highly flexible helical linker region that allows CaM to adopt a variety of conformations when bound to different targets. Each lobe of CaM contains a pair of EF- hand motifs allowing it to bind four Ca^{2+} ions, and saturation of CaM with Ca^{2+} induces a conformational change that permits the protein to interact with and activate a surprisingly diverse set of target enzymes. When intracellular Ca^{2+} concentration increases, it binds to CaM. The CaM can activate myosin light chain kinase. Activated myosin light chain kinase phosphorylates the regulatory light chain of myosin, allowing for the activation of myosin ATPase. The ATP provides the energy source needed for the cross-bridging cycles between actin and myosin. These cross-bridging interactions constitute cellular contraction [74, 75] and concerted contraction of PASMCs, pulmonary vasoconstriction.

Calcium homeostasis in vascular smooth muscle

Intracellular Ca^{2+} concentration can be caused by release of Ca^{2+} from internal storage sites, such as sarcoplasmic reticulum (SR) or influx of Ca^{2+} from extracellular fluid through L-type voltage-dependent Ca^{2+} channels (VDCCs), receptor-operated Ca^{2+} channels (ROCCs), or store-operated Ca^{2+} channels (SOCCs) (**Figure 2**). VDCCs are the main Ca^{2+} channel in the vascular smooth muscle cell membrane and can be activated by membrane depolarization and blocked by Ca^{2+} channel blockers such as nifedipine and verapamil. ROCCs are in many types of smooth muscle and can be activated by inositol lipid signaling which is one of most widespread signal transduction cascades.

Store-operated calcium entry plays a very important role in refilling Ca^{2+} in SR and maintaining Ca^{2+} homeostasis in PASMCs. Activation

of this pathway can be independent of IP_3 production, since various procedures that deplete internal stores (thapsigargin and CPA) are able to stimulate Ca^{2+} entry across the plasma membrane without affecting the intracellular IP_3 level. SOCE in smooth muscle cell can be observed in two ways. First, SOCE is observed as a sharp rise in intracellular Ca^{2+} concentration occurring right after passive store depletion with CPA. In the absence of extracellular Ca^{2+}, CPA, by blocking Ca^{2+} sequestration into the SR, induces a transient rise in intracellular Ca^{2+} concentration due to leakage of Ca^{2+} from the SR. The CPA-induced intracellular Ca^{2+} concentration rise declines back to the original baseline level after 5-10 min as the SR Ca^{2+} is depleted. Under these conditions, restoration of extracellular Ca^{2+} induces a further rise in intracellular Ca^{2+} concentration due to SOCE, which is inhibited reversibly by the SOCC blockers such as SKF-96365 and Ni^{2+}. Second, SOCE can be evaluated by monitoring Fura-2 fluorescence excited at 360 nm before and after addition of $MnCl_2$ (200 μM) to the cell perfusate. It was evaluated from the rate at which Fura-2 fluorescence was quenched by Mn^{2+}, which entered the cell as a Ca^{2+} surrogate and reduced Fura-2 fluorescence upon binding to the dye. Fluorescence excited at 360 nm was the same for Ca^{2+}-bound and Ca^{2+}-free Fura-2; therefore, changes in fluorescence can be assumed to be caused by Mn^{2+} alone [76].

Voltage-dependent Ca^{2+} influx pathway

VDCCs are a group of voltage-gated ion channels found in excitable cells with permeability to the ion Ca^{2+} [77, 78]. These channels are slightly permeable to sodium ions, so they are also called Ca^{2+}-Na^+ channels, but under normal physiological conditions their permeability to calcium is about 1000-fold greater than to sodium. VDCCs are normally closed at resting membrane potential. They are activated at depolarization of membrane potential. The activity of K^+ channels in the membrane is thus important for the regulation of resting membrane potential and plays an important role in vascular contractility. Kv channels, the most diverse group of K^+ channels, are ubiquitously expressed in vascular smooth muscle cells [79, 80]. When Kv channels close, the membrane depolarizes, which leads to increased intracellular Ca^{2+} concentration by inducing Ca^{2+} influx through VDCCs. Inhibition of Kv channels with 4-aminopyridine reduces whole cell K^+ currents, causes membrane depolarization, and results in increased intracellular Ca^{2+} concentration in PASMCs. In isolated pulmonary arterial rings, inhibition of Kv channels by 4-aminopyridine increases isometric tension as a result of PASMC contraction and vasoconstriction in response to membrane depolarization and Ca^{2+} influx through VDCC.

VDCCs are formed as a complex of several different subunits: α1, α2δ, β1-4 and v. The α1 subunit forms the ion conducting pore while the associated subunits have several functions including modulation of gating [81]. VDCC can be divided into six different subtypes based on their functional characteristics [82-84]. However, in PASMCs L- and T- type channels are the important channels for voltage-gated Ca^{2+} entry involved in excitation-contraction coupling and cell proliferation [85, 86]. The L- type VDCC is activated by high voltage, whereas inactivation is slow. The T- type channels are activated by low voltage, whereas inactivation is much faster than L- type channels. L- type calcium channels are also enriched in the t-tubules of striated muscle cells, including skeletal and cardiac myofibers. When these cells are depolarized, the L- type calcium channels open as in smooth muscle. Ca^{2+} is released from the SR and is able to bind to troponin C on the actin filaments. The muscles then contract through the sliding filament mechanism, causing shortening of sarcomeres and muscle contraction.

Receptor- and store-operated Ca^{2+} influx pathways

In 1986, based on a series of experiments in parotid acinar cells investigating the relationship between Ca^{2+} release from internal stores, Ca^{2+} entry, and store refilling, the concept of store operated Ca^{2+} entry was first proposed [33]. Stimulation of membrane receptors, such as GPCRs and receptor tyrosine kinases (RTKs), by their extracellular ligands results in the activation of phospholipase C and the production of two important second messengers, diacylglycerol (DAG) and inositol 1, 4, 5-trisphosphate (IP_3). DAG can then open ROCC, leading to Ca^{2+} influx and increased intracellular Ca^{2+} concentration. This process is called as receptor-operated Ca^{2+} entry (ROCE). Additionally, IP_3 stimu-

lates the IP_3 receptor (IP_3R). IP_3R is a Ca^{2+} release channel on SR/ER membrane, to release Ca^{2+} from the SR/ER to the cytosol. This leads to a depletion or a reduction of the SR/ER Ca^{2+} store. After depletion of Ca^{2+} from the SR/ER, a Ca^{2+} deficiency signal is transmitted to SOCC on the plasma membrane causing SOCC open and allows Ca^{2+} to flow into cytosol, this process is referred to as store-operated Ca^{2+} entry (SOCE).

Putney et al first described SOCE, and referred to then as capacitative Ca^{2+} entry [87]. The SOCC is believed to be composed of mammalian homologs of transient receptor potential (TRP) proteins and, in the case of store-operated Ca^{2+} channels, may complex with the recently identified Orai and STIM1 (stromal interacting molecule 1) proteins. The exact molecular identity of the proteins encoding SOCC remains under investigation, although isoforms in the canonical TRP (TRPC) subfamily are the leading candidates. Based on research on Drosophila phototransduction, a transient receptor potential (TRP) gene encoding a subunit of a Ca^{2+}-permeable channel which was identified in 1969, was thought to be involved in store operated Ca^{2+} entry. There are seven related members of the transient receptor potential channel (TRPC) family, designated TRPC1-7 (the numbering reflects the order of their discoveries) [88, 89]. We and Golovina VA et al [43] have demonstrated that STIM1 and TRPC proteins are expressed in PASMCs. Most labs have shown that only TRPC1 and TRPC6 are highly abundant in PASMCs [24, 25, 90, 91], and some labs reported that TRPC3 [25] and TRPC4 [24, 90] are also expressed in PASMCs.

Effect of chronic hypoxia on Ca^{2+} channels

Development of chronic hypoxic pulmonary hypertension is associated with elevated resting intracellular Ca^{2+} concentration in PASMCs and contraction of pulmonary vascular smooth muscle. It has been wildly accepted that the maintenance of increased PASMC intracellular Ca^{2+} concentration and tone during CH requires Ca^{2+} influx through pathways other than VDCCs. TRPC proteins play very important roles in response to chronic hypoxia. The expression of TRPC1 and TRPC6, but not TRPC4 in pulmonary vascular smooth muscle from chronically hypoxic rats increased, compared with the control from normoxic rats [24].

Compared with Kv channels, little is known about the roles and functions of TRPC proteins as well as its upregulation mechanisms in the development of chronic pulmonary hypertension. Yu et al was the first to demonstrate the role for HIF-1 in mediating the pulmonary responses to CH. The research data showed that the development of right ventricular hypertrophy, and vascular remodeling was delayed in Hif1α+/- compared with Hif1α+/+ mice [92]. We first demonstrated that previous observations of elevated intracellular Ca^{2+} concentration and SOCE in transiently-cultured PASMCs from chronically hypoxic rats for 21 days. We also showed that the increases in basal intracellular Ca^{2+} concentration and TRPC1/6 expression are absent in Hif1α+/- mice. Accordingly, overexpression of Hif1α also increases the expression of TRPC1/6, but not TRPC4 [24].

Additional evidence for the involvement of TRPC-related channels under hypoxic condition comes from the study of mice lacking TRPC6. TRPC6 knockout mice have no pulmonary vascular reactivity to hypoxia although they fully respond to non-hypoxia induced vasoconstriction. PASMCs isolated from TRPC6-deficient mice exhibit no elevated intracellular Ca^{2+} concentration and membrane current when exposed to hypoxia, in contrast to wild-type PASMCs [93]. TRPC6 may modulate intracellular calcium and membrane potential by subsequent gating of L-type calcium channels and Kv.

Summary

Sustained pulmonary vasoconstriction and excessive pulmonary vascular remodeling are the major causes for the elevated pulmonary vascular resistance. Although previous studies have characterized some of the functional changes that occur in the pulmonary vasculature in response to CH, the cellular mechanisms of this disease is still under investigation. In this review, we and others have found a very important role for alteration in PASMC function during CH. The increases in intracellular Ca^{2+} concentration is the key factor responsible for both changes transient or reversible and changes permanent or irreversible. The function of ion channels involved in the former process, including Kv channels, VDCCs and SOCCs, will have been changed, either “turned on” or “turned off”. Compared with Kv chan-

nels, little is known about the roles and functions of TRPC proteins as well as their upregulation mechanisms in the development of chronic pulmonary hypertension, despite the fact that the expression of TRPC proteins especially TRPC1 and TRPC6 has been confirmed. We and others also found that HIF-1 plays a very important role in mediating the physiological responses to hypoxia and development of pulmonary hypertension. Elucidating the factors involved in this disease process will lead to improved methods of prevention and treatment of this lethal complication of this disease.

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Disclosure of conflict of interest

None.

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