The Role of Classical Transient Receptor Potential Channels in the Regulation of Hypoxic Pulmonary Vasoconstriction

B. Fuchs, A. Dietrich, T. Gudermann, H. Kalwa, F. Grimminger, and N. Weissmann

Abstract Hypoxic pulmonary vasoconstriction (HPV) is an essential mechanism of the lung matching blood perfusion to ventilation during local alveolar hypoxia. HPV thus optimizes pulmonary gas exchange. In contrast chronic and generalized hypoxia leads to pulmonary vascular remodeling with subsequent pulmonary hypertension and right heart hypertrophy. Among other non-selective cation channels, the family of classical transient receptor potential channels (TRPC) has been shown to be expressed in pulmonary arterial smooth muscle cells. Among this family, TRPC6 is essential for the regulation of acute HPV in mice. Against this background, in this chapter we give an overview about the TRPC family and their role in HPV.

Keywords Hypoxia • lung • TRPC • pulmonary arterial smooth muscle cells

1 Introduction

Hypoxic pulmonary vasoconstriction (HPV) is a physiological lung mechanism that directs blood perfusion from poorly ventilated to well-ventilated lung areas to optimize gas exchange.[1] In contrast to the systemic circulation, in which hypoxia leads to vasodilation, HPV is unique to the pulmonary vasculature[2] (Fig. 12.1a) and is triggered by mild hypoxia (alveolar $pO_2 < 100$ mmHg).[1] A disturbance of this mechanism may result in life-threatening hypoxemia[3] (Fig. 12.1b). Furthermore, when hypoxia is generalized and chronic, as in many lung diseases (e.g., chronic obstructive pulmonary disease [COPD], pneumonia, fibrosis) or in residents at high

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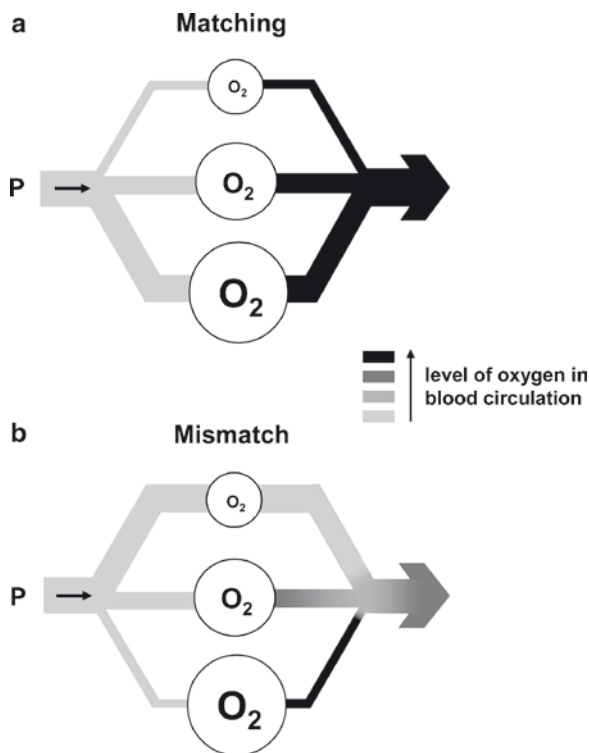
Fig. 12.1 Ventilation-perfusion matching by hypoxic pulmonary vasoconstriction (HPV) in the lung under (patho)physiological conditions (P = blood flow). (**a**) Under physiological conditions, perfusion is matched to the alveolar oxygen partial pressure (pO_2) by HPV. Reproduced with permission.[9] (**b**) Under disturbed HPV, a mismatch of blood flow and alveolar ventilation may result in hypoxemia. The blood perfusion color indicates the oxygen concentration of the blood in a graded manner: the darker the color, the higher the oxygen concentration. *Large circles* high alveolar pO_2, *small circles* low alveolar pO_2

altitude, the subsequent pulmonary vasoconstriction leads to chronic pulmonary hypertension, vascular remodeling, and right heart failure.[4,5]

Although the physiological function of HPV was originally described in 1946 by Euler and Liljestrand, the underlying oxygen-sensing and signal transduction processes remain unclear.[6] Several hypothetical mechanisms for the O_2-sensing in pulmonary artery smooth muscle cells (PASMC) are currently proposed, for the most part related to reactive oxygen species (ROS).[7] Nicotinamide adenine dinucleotide phosphate-oxidase (NAD(P)H) oxidases, similar to that found in neutrophils, as well as mitochondria are strongly suggested to affect superoxide generation during hypoxia. However, it is unclear whether ROS generation is increased or decreased under hypoxic conditions. Moreover, there is also evidence for a hypoxia-induced increase of cyclic adenosine diphosphate-ribose (cADPR), resulting in a rise of intracellular Ca^{2+} ($[Ca^{2+}]_i$) as well as a role for cytochrome P450-dependent processes in HPV.[1]

The smooth muscle layer of the precapillary vessels has been identified as the effector cell type. Moreover, isolated PASMCs respond to acute hypoxia by an increase of the $[Ca^{2+}]_i$ concentration and subsequent contraction, indicating that these cells function as sensor as well as effector cells.[8]

In addition to L-type voltage-operated Ca^{2+} channels (VOCC), nonselective cation channels have been identified as important players in the regulation of vascular tone by their role in mediating the entry of cations like Ca^{2+} and Na^+.[9] Among other nonselective cation channels, the family of classical transient receptor potential (TRPC) channels has been shown to be expressed in pulmonary arterial smooth muscle[10–12] and to play a major role in the regulation of HPV under acute hypoxia.[5,13,14]
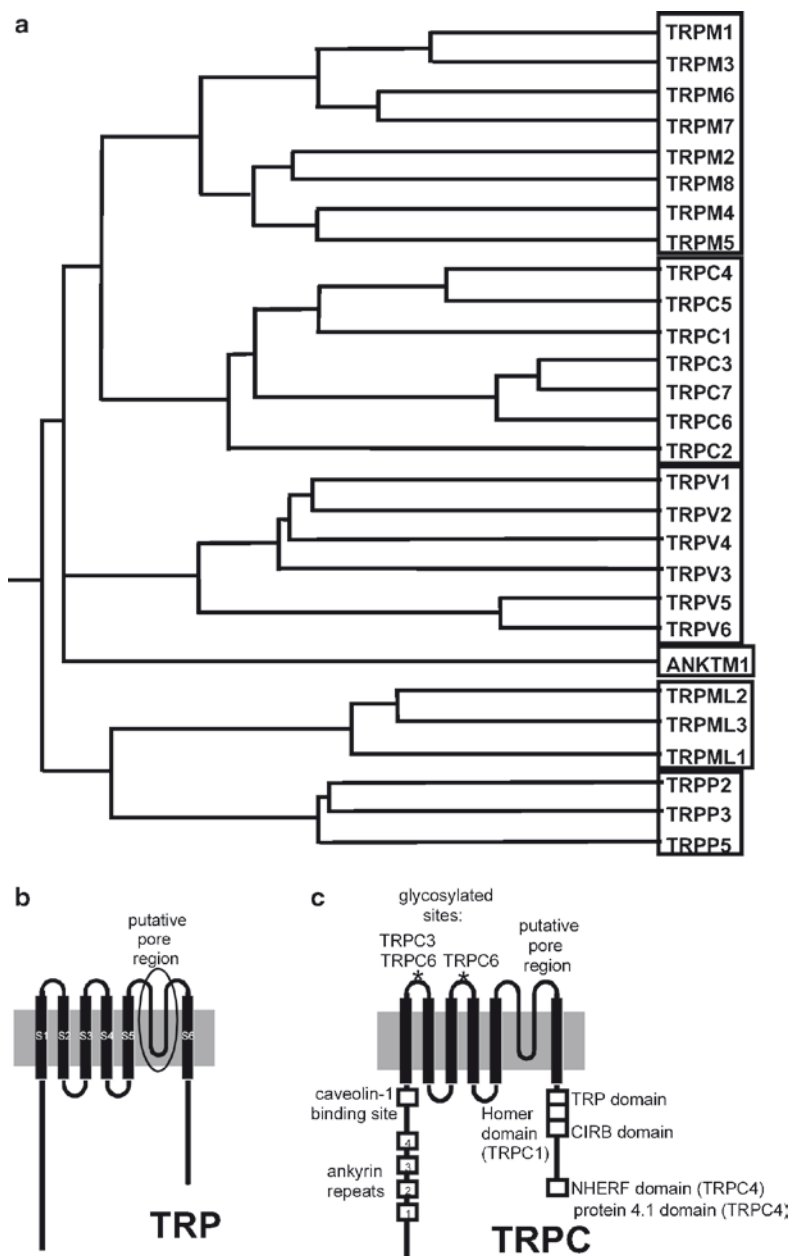
2 The Classical Transient Receptor Potential Family of Nonselective Cation Channels

2.1 Introduction

Transient receptor potential (TRP) channels were first discovered in the fruit fly *Drosophila melanogaster* and constitute a superfamily of cation channels. In contrast to vertebrates, the transduction of visual stimuli in *Drosophila melanogaster* is a phospholipase C-dependent process that leads to an activation of membrane channels with subsequent membrane depolarization.[15,16] Interestingly, *Drosophila melanogaster* with a mutation in the *trp* locus exhibited a transient instead of a sustained response to light due to a defect in Ca^{2+} influx following the initial Na^+ influx. The channels responsible for this light-induced Ca^{2+} influx were named TRP channels.[17,18]

2.2 Identification and Structural Properties of Mammalian TRP Channels

The first mammalian TRP channel closely related to *Drosophila melanogaster* TRP channels was identified in 1995 and founded a new TRP family called the classical or canonical TRP (TRPC) channels.[19] In the ensuing years, six other TRPC family members and other TRP families, such as TRPM (for melastatin), TRPV (for vanilloid receptor), TRPP (for polycystic kidney disease (PKD) proteins), TRPML (for mucolipidins), and TRPA (for ankyrin-rich proteins) were identified. Figure 12.2a shows the phylogenetic tree of the TRP superfamily.[15] The classification of the mammalian TRP proteins in these six groups results from their structure (≥90% amino acid similarity within each group), whereas the characteristic features of TRP proteins in general are the six transmembrane domains (S1–S6), cytoplasmic N- and C-termini, and a hydrophobic loop between S5 and S6, as well as cation permeability[15] (Fig. 12.2b). Thus, the channel pore is thought to be formed

Fig. 12.2 Phylogenetic tree and plasma membrane topology of the TRP proteins. (**a**) Phylogenetic tree of the TRP superfamily subdivided into six families according to their amino acid homology (≥90% within each group).[15] (**b**) Plasma membrane topology of TRP proteins: six transmembrane domains (S1–S6) with a hydrophobic putative pore region between S5 and S6 and the cytoplasmic N- and C-termini.[15] (**c**) Plasma membrane topology and functional domains of TRPC channels. TRPC channels have typically four ankyrin repeats, a caveolin-1-binding site, and a TRPC domain as well as a calmodulin IP$_3$-receptor-binding domain (CIRB). In addition, there is one glycosylation site in TRPC3, two glycosylation sites in TRPC6, a protein 4.1-binding domain, and an Asparagin-Histidin-Glutamat-Arginin-Phenylalanin-binding domain in TRPC4, as well as a homer domain in TRPC1. Reproduced with permission[9]

by the loop between S5 and S6. TRP channels are homo- or heterotetramers with members of the same subfamily[15] and are permeable to Na^+, K^+, Cs^+, Li^+, Ca^{2+}, Mn^{2+}, and Mg^{2+} after voltage-independent activation.[20]

2.3 Characteristics of the TRPC Channel Subfamily

The seven members of the TRPC family share common structural features in addition to the six transmembrane-spanning domains (S1–S6) and the putative pore region between S5 and S6 as the typical TRP structure: four N-terminal ankyrin repeats as well as a TRP box (amino acid sequence EWKFAR) and a calmodulin inositol 1,4,5-trisphosphate (IP_3)-receptor-binding (CIRB) site in the C-terminal tail. Other protein-binding domains vary between the different TRPC proteins (Fig. 12.2c). The TRPC family can be divided into three subfamilies on the basis of their amino acid homology: TRPC1, TRPC4/5, and TRPC3/6/7. TRPC2 is a pseudo-gene in humans but plays an important role in the sexual recognition of mice.[9,21]

Whereas TRPC4 and TRPC5 share approximately 65% of the amino acid sequence, the members of the TRPC3/6/7 subfamily form a structural and functional subfamily with 70–80% homology of the amino acid sequence and direct activation by diacylglycerol (DAG).[9] DAG production results from activation of G protein-coupled receptors or receptor tyrosine kinases and subsequent activation of phospholipase C isoforms (PLCβ or PLCγ, respectively), leading to hydrolysis of phosphatidylinositol 4,5-bisphosphate.[22] While DAG mediates cation entry through receptor-operated channels (ROCs), IP_3, the second product of this signal cascade, induces Ca^{2+} depletion of the endoplasmatic reticulum (ER), which subsequently activates Ca^{2+} influx from the extracellular space through store-operated channels (SOCs), also called capacitative Ca^{2+} entry (CCE). SOCs are discussed as mechanisms for TRPC1, 4, and 5 as well as for TRPC3 and 7.[9,23] The varying function of TRPC3 and TRPC7 as ROCs or SOCs is probably caused by the formation of heterotetrameric channels as a consequence of the interaction with other TRPC isoforms.[20] Thus, TRPC channels can be gated by different stimuli, leading to multisided channel activation.[24] In general, the properties of heteromultimer proteins depend on their protein composition. Furthermore, the function of TRPC channels may also depend on many additional modulators, making the mechanisms more complex.[21]

2.4 Expression Pattern and Function of TRPC Channels

After identification of the TRPC channel subfamily, the expression levels of TRPC family members in different organs and cell types, as well as their functional roles, have been intensely investigated.

TRPC1 exhibits a widespread, but not ubiquitous, expression in different cell types, whereas an important role for vascular smooth muscle has been suggested that is related to the contractile and proliferative functions of muscle. However, there is evidence that

TRPC1 only contributes to a heterotetrameric ion channel complexed with other TRPC isoforms. Heterologous expression studies and biochemical investigations indicated a possible association of TRPC1 with TRPC4, TRPC5, TRPC3, and TRPP2.[15,24] This interaction with other TRPC isoforms seems to be required for the translocation of TRPC1 to the plasma membrane, as shown in TRPC1–TRPC4 coexpression studies.[20]

TRPC3 is highly expressed in brain as well as cardiac and smooth muscle cells (SMCs) and has been detected in the slow oxidative myofibers in skeletal muscle after neuromuscular activity.[15] Numerous functions of TRPC3 have been suggested in agonist-induced contraction in SMCs.[24] Interestingly, TRPC3 has high basal activity, which is decreased after the addition of a second glycosylated site at the extracellular portion of the channel, as found in TRPC6.[15] Moreover, basal and agonist-induced cation influx into SMCs as well as smooth muscle contractility were reportedly increased after the replacement of TRPC6 by TRPC3 in TRPC6-deficient (TRPC6$^{-/-}$) mice.[25] Thus, protein glycosylation seems to play an important role for channel activity. TRPC3 also forms functional heteromultimeric channels with TRPC6.[24]

TRPC4 is particularly expressed in the endothelium, where it regulates microvascular permeability, endothelium-dependent vasorelaxation of SMCs, and gene transcription. In SMCs, TRPC4 regulates cell proliferation and contraction.[20] TRPC4 as well as TRPC5 are reportedly thought to be assisted in their function as nonselective cation channels by TRPC1.[15]

TRPC5 was first found to be primarily expressed in the brain, but there are conflicting reports on the expression of TRPC5 in the pulmonary vasculature. Since TRPC5 is only poorly characterized, the role of this channel is unclear.[15,20]

TRPC6 is expressed in many tissues rich in SMCs but is most prominently expressed in lung tissue. Many studies suggest an important role of TRPC6 in vascular and pulmonary SMCs.[15] Investigations in TRPC6$^{-/-}$ mice in comparison to wild-type (WT) mice have also revealed a unique role for TRPC6 in the regulation of airway and vascular smooth muscle contractility.[25,26] Most interestingly, small precapillary pulmonary arteries, in contrast to large pulmonary arteries, do not express TRPC3.[9] Besides a role for TRPC6 for SMC contraction, there is evidence for a role of TRPC6 in cell proliferation.[24]

TRPC7 was first identified in mouse brain, but expression is also found in SMCs from the aortic and renal arteries as well as in endothelial cells from the cerebral and coronary arteries.[20] TRPC7 expression is also reported in the eye, spleen, and testis.[15]

3   Role of the TRPC Channels in Acute Hypoxic Pulmonary Vasoconstriction

3.1   Role of Ca^{2+}Channels in Acute HPV

Under physiological conditions, acute alveolar hypoxia leads to vasoconstriction of the precapillary arteries in the lung. Therefore, a rise of $[Ca^{2+}]_i$ in PASMCs is a key event in this process, inducing Ca^{2+}/calmodulin-dependent activation of

myosin light chain kinase, phosphorylation of the myosin light chains, actin-myosin interactions, and contraction.[13,27] However, the regulation of $[Ca^{2+}]_i$ has not yet been resolved. In general, $[Ca^{2+}]_i$ can be increased by Ca^{2+} influx from the extracellular space or by release from intracellular Ca^{2+} stores. At least three classes of Ca^{2+}-permeable channels in the plasma membrane are known: the L-type VOCCs, which are regulated by the resting membrane potential; the ROCs, which are activated by agonists; and SOCs, which are opened by depletion of Ca^{2+} from the sarcoplasmic reticulum (SR).[4,28] Concerning the Ca^{2+} release from intracellular Ca^{2+} stores, IP_3 receptor-mediated Ca^{2+} release from IP_3-sensitive SR as well as ryanodine receptor-mediated Ca^{2+} release from ryanodine-sensitive SR are known.[29]

One well-documented concept for the regulation of HPV proposes that a hypoxia-induced inhibition of voltage-gated K^+ (K_v) channels leads to membrane depolarization and Ca^{2+} entry through VOCCs.[30] However, since antagonists of K_v channels did not block HPV and VOCC antagonists exhibited no or only partial prevention of the hypoxic response, the mechanism of HPV seems to be more complex.[12,13] Growing evidence in the literature indicates a role for hypoxia-induced Ca^{2+} release from intracellular stores activating SOCs and CCE, possibly in addition to VOCCs.[13,14,30] Thus, depletion of intracellular Ca^{2+} stores, and thereby activation of SOCs by cyclopiazonic acid (CPA) and simultaneous application of nifedipine to prevent Ca^{2+} influx through VOCCs, was shown to cause an increase of $[Ca^{2+}]_i$ that was markedly enhanced under hypoxic compared to normoxic conditions.[13,14,30] Moreover, the pharmacological agents SKF-96365, Ni^{2+}, and La^{3+}, which block influx through nonselective cation channels in PASMCs, were potent inhibitors of HPV at concentrations that did not affect VOCCs. This is the first direct evidence that nonselective cation channels may play an important role in HPV.[14] Nevertheless, the VOCC inhibitor nifedipine was quite effective in preventing and reversing HPV, suggesting a hypoxia-induced influx through both SOCCs and VOCCs.[14]

Nonselective cation channels, which are associated with both SOCs and ROCs, are reported to be likely formed of homo- or heteromultimers of TRP proteins.[31,32] Among the TRP channels, especially TRPC proteins are expressed in SMCs of distal pulmonary arteries, which are suggested to be O_2 sensor and effector cells, at least of acute HPV.[11] However, less is known about the functional role of TRPC channels in the pulmonary circulation.

3.2 Importance of TRPC6 Channels in Acute HPV

TRPC6 is highly expressed in lung tissue as well as pulmonary and vascular SMCs.[23] Since there is a lack of specific TRPC channel blockers, a TRPC6$^{-/-}$ mouse model was developed by gene inactivation in embryonic stem cells to investigate
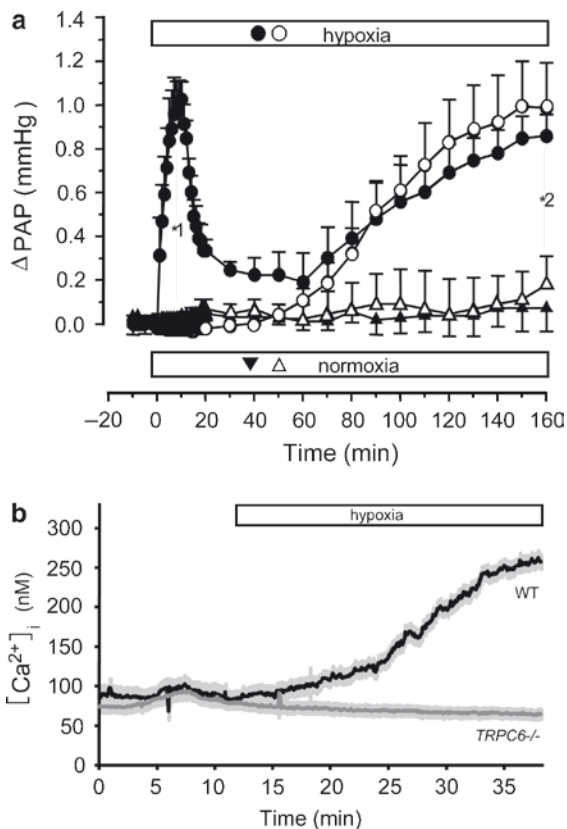
Fig. 12.3 Involvement of TRPC6 in acute hypoxic pulmonary vasoconstriction. (**a**) Time course of the increase in pulmonary arterial pressure (ΔPAP) in isolated, buffer-perfused, and ventilated mouse lungs (*filled circles* WT; *open circles* TRPC6$^{-/-}$) during 160 min of hypoxic ventilation (1% O$_2$). Control lungs were ventilated normoxically (*filled triangles* WT; *open triangles* TRPC6$^{-/-}$). $_*$1 indicates a significant difference ($p < 0.05$) between WT and TRPC6$^{-/-}$ mice after applying acute hypoxia; $_*$2 indicates significant differences ($p < 0.05$) between normoxic (WT and TRPC6$^{-/-}$) and hypoxic (WT and TRPC6$^{-/-}$) mice. (**b**) Time course of [Ca^{2+}]$_i$ in primary cultured PASMCs from WT and TRPC6$^{-/-}$ mice on exposure to hypoxia. Cells were loaded with fura-2 and analyzed by single-cell fluorescence imaging. Horizontal bars indicate endothelin priming (4 n*M* ET-1) and hypoxic perfusion (hypoxia) of PASMCs. Reproduced with permission[5]

functional parameters in comparison to WT mice.[22,25] Interestingly, TRPC6$^{-/-}$ mice showed increased vascular smooth muscle contractility, suggesting a critical role for TRPC6 in regulating vascular smooth muscle tone.[25]

To assess the role of TRPC6 in HPV, we analyzed the pressor response in isolated ventilated and perfused lungs from WT and TRPC6$^{-/-}$ mice during acute (<20 min) and sustained (60 – 160 min) hypoxia.[5] As shown in Fig. 12.3a, ventilation of lungs from WT mice with 1% O$_2$ provoked a biphasic profile of pulmonary arterial

pressure (PAP), with a first transient increase followed by a second progressive increase of PAP. Interestingly, the acute phase of HPV was completely absent in TRPC6$^{-/-}$ mice, while the sustained phase was not significantly different compared to WT mice. Thus, the general muscular contractility was not affected, the vasoconstriction induced by the thromboxane mimetic U46619 being unchanged.[5] Under normoxic conditions, the PAP did not differ between WT and TRPC6$^{-/-}$.[5] These results clearly show the indispensable role of TRPC6 in acute HPV as well as the differential regulation of the acute and sustained phase of HPV. Moreover, since partial occlusion of alveolar ventilation provoked severe hypoxemia in TRPC6$^{-/-}$ mice but not in WT mice, the profound physiological relevance of TRPC6 in acute HPV was confirmed.[5]

3.3   Role of TRPC6 Channels in the Increase of Intracellular Ca^{2+} Concentration in Acute HPV

To investigate the cellular mechanism of the TRPC6 dependency of HPV, alterations of [Ca^{2+}]$_i$ were investigated in hypoxia-incubated PASMCs from TRPC6$^{-/-}$ and WT mice, using an established method based on fluorescence imaging of single cells loaded with the fluorescent dye fura-2.[33,34] The expression level of the TRPC subtypes was unchanged in the PASMCs from TRPC6$^{-/-}$ mice except for TRPC6, as expected.[5] In contrast to WT PASMCs, the hypoxia-induced rise of [Ca^{2+}]$_i$ after priming with endothelin 1 (ET-1) was completely absent in PASMCs from TRPC6$^{-/-}$ (Fig. 12.3b).[5] The "priming" with a low dose of vasoactive agents such as angiotensin II, ET-1, or prostaglandin F2α turned out to be a prerequisite for HPV.[35–37]

Interestingly, the observed increase of [Ca^{2+}]$_i$ in WT PASMCs was completely dependent on extracellular Ca^{2+}, although ET-1 caused a rise of [Ca^{2+}]$_i$ in both cell types in the absence of extracellular Ca^{2+}.[5] This finding challenges the suggested contribution of SOCs and CCE to the regulation of HPV[12–14] and may be related to the fact that PASMCs of the precapillary resistance vessels were investigated in this study.

However, since the potent blocker of VOCC nicardipine almost completely inhibited Ca^{2+} entry in WT PASMCs and acute HPV in isolated lungs, the increase of [Ca^{2+}]$_i$ appears to be mostly attributable to VOCCs.[5] Then, what role do TRPC6 channels play? As described in the literature, TRPC6 channels are predominantly permeable to Na$^+$, and only a small percentage of the whole-cell current is caused by Ca^{2+} in the presence of extracellular Na$^+$.[38] In contrast to VOCCs, TRPC6 channels are permeable for Mn^{2+}. Thus, the hypoxia-induced influx of non-Ca^{2+} ions through TRPC6 channels was analyzed by a method called Mn^{2+} quenching. These experiments showed that hypoxia induced an increase in the Mn^{2+} quenching rate as a result of increased Mn^{2+} influx in WT but not in TRPC6$^{-/-}$ PASMCs.[5] This result is in line with the concept that Na$^+$ influx through TRPC6 channels leads to membrane depolarization and activation of VOCCs.[38–40] Moreover, an increase of

intracellular Na^+ has been shown to block the K_v channels[41] known to activate VOCCs. The important role of K_v channels was demonstrated by the impairment of HPV in mice lacking the K_v channel.[4,42]

3.4   Activation of TRPC6 Channels in Acute HPV

The receptor-operated, store-independent TRPC6 channel was the first ion channel shown to be DAG activated in a membrane-delimited fashion, independently of protein kinase C. However, the exact location of the DAG-binding site in the TRPC6 protein is still unresolved. In addition, sensitivity to the arachidonic acid metabolite 20-hydroxyeicosatetraenoic acid (HETE) as well as an activating effect by Ca^{2+}/calmodulin or protein phosphorylation have been reported.[23]

Since the recombinant TRPC6 channels heterologously expressed in human embryonic kidney (HEK) 293 cells were not activated by hypoxia, a direct activation of TRPC6 channels by hypoxia could be excluded.[5] Interestingly, a fluorescent DAG sensor expressed in PASMCs revealed the localization of DAG in the cytosol under normoxic conditions. Under hypoxia, DAG was translocated to the plasma membrane, suggesting gating of TRPC6 via DAG. In addition, a DAG kinase inhibitor activated the TRPC6 channels, supporting the hypothesis of hypoxia-induced DAG accumulation mediated by inhibition of DAG kinase or phospholipases.[5] Speculatively, the activation of TRPC6 in HPV may occur via ROS as it has been proposed that the O_2-sensing mechanism underlying HPV involves ROS.[5] NAD(P)H oxidase isoforms, different from those found in neutrophils, as well as mitochondria have been shown to be involved in the regulation of HPV in this regard. However, it is still unclear whether ROS generation is increased or decreased under hypoxic conditions.[1] Moreover, a role for a decreased adenosine monophosphate/adenosine triphosphate ratio as well as a role for cytochrome P450-dependent processes have been suggested for HPV.[1,43]

3.5   Hypothesis of the Mechanism of Acute HPV

A hypothetic model of the signal transduction pathway in PASMCs underlying acute HPV is described in Fig. 12.4. Initial priming of ET-1 activates PLC, producing a basal DAG concentration without activation of TRPC6 itself, but which is required for hypoxia-induced TRPC6 activation. Hypoxia-induced DAG accumulation results from the activation of PLCs[1] or phospholipase D (PLD)[2] or inhibition of DAG-degrading DAG kinases,[3] probably induced by changes in ROS production, and ultimately leads to Na^+ influx through TRPC6 channels.
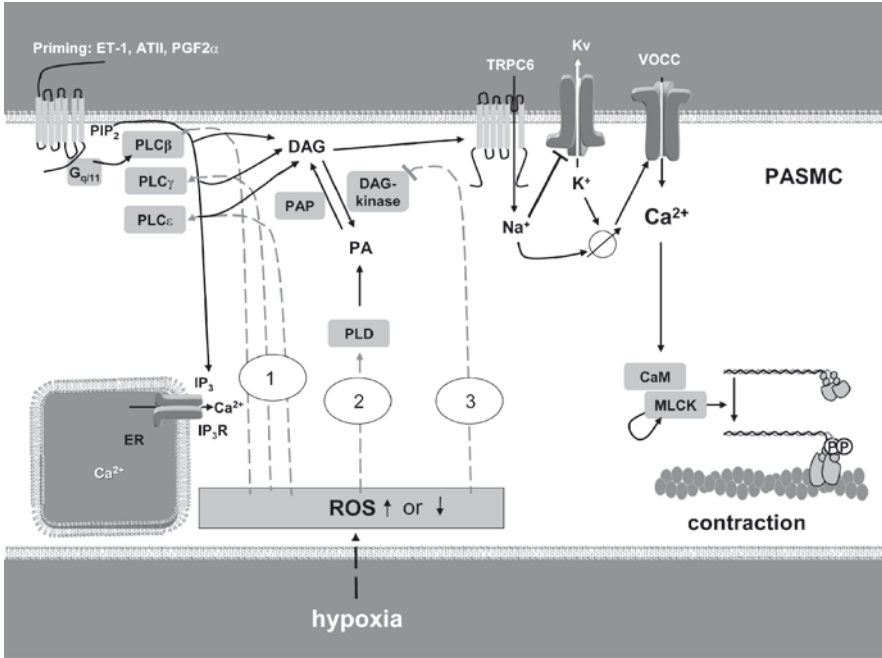
Fig. 12.4 Hypothetical model of the signal transduction pathway underlying acute hypoxic pulmonary vasoconstriction in PASMCs. According to this model, hypoxia-induced diacylglycerol (DAG) accumulation causes a cation influx through TRPC6. The DAG increase may be caused by activation of either phospholipase C (1), phospholipase D (2) or by inhibition of DAG-degrading DAG kinases (3). *ET-1* endothelin-1; *ATII* angiotensin II; *PGF2α* prostaglandin F2α; *Gq/11* G protein type q and 11; *PLC* phospholipase C; *PIP₂* phosphatidylinositol 4,5-bisphosphate; *IP₃* inositol-1,4,5 trisphosphate; *VOCC* voltage gated calcium channel; *ER* endoplasmatic reticulum; *IP₃-R* IP₃-receptor; *PLD* phospholipase D; *ROS* reactive oxygen species; *PA* phosphatidic acid; *PAP* phosphatidic acid phosphatase; *CaM* calmodulin; *MLCK* myosin-light chain kinase. Reproduced with permission[9]

A subsequent membrane depolarization, probably also caused by Na$^+$-induced inhibition of K$_v$ channels, activates Ca^{2+} influx through VOCCs, resulting in contraction of the PASMCs.

4 Conclusion

HPV is an important mechanism in the lung that has been under investigation for more than 60 years. The nonselective cation channel TRPC6 has been identified as playing an essential role in this mechanism, as demonstrated by a complete absence of the acute response to hypoxia in mice lacking this ion channel.

Therefore, the TRPC6 channels offer a promising therapeutic target for pharmacological intervention in the control of pulmonary hemodynamics and gas exchange.

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