Published in final edited form as:

Compr Physiol. 2011 July ; 1(3): 1555–1602. doi:10.1002/cphy.c100023.

Endothelial and Smooth Muscle Cell Ion Channels in Pulmonary Vasoconstriction and Vascular Remodeling

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Abstract

The pulmonary circulation is a low resistance and low pressure system. Sustained pulmonary vasoconstriction and excessive vascular remodeling often occur under pathophysiological conditions such as in patients with pulmonary hypertension. Pulmonary vasoconstriction is a consequence of smooth muscle contraction. Many factors released from the endothelium contribute to regulating pulmonary vascular tone, while the extracellular matrix in the adventitia is the major determinant of vascular wall compliance. Pulmonary vascular remodeling is characterized by adventitial and medial hypertrophy due to fibroblast and smooth muscle cell proliferation, neointimal proliferation, intimal, and plexiform lesions that obliterate the lumen, muscularization of precapillary arterioles, and *in situ* thrombosis. A rise in cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{cyt}}$) in pulmonary artery smooth muscle cells (PASMC) is a major trigger for pulmonary vasoconstriction, while increased release of mitogenic factors, upregulation (or downregulation) of ion channels and transporters, and abnormalities in intracellular signaling cascades are key to the remodeling of the pulmonary vasculature. Changes in the expression, function, and regulation of ion channels in PASMC and pulmonary arterial endothelial cells play an important role in the regulation of vascular tone and development of vascular remodeling. This article will focus on describing the ion channels and transporters that are involved in the regulation of pulmonary vascular function and structure and illustrating the potential pathogenic role of ion channels and transporters in the development of pulmonary vascular disease.

Introduction

The lungs are the only organ in the body that receives the entire cardiac output (CO) at all time. Pulmonary circulation is normally a high-flow, low-resistance, low-pressure system that carries blood into the pulmonary capillaries where the venous blood takes up oxygen and unloads excess carbon dioxide. The most serious chronic disorder of the pulmonary circulation is pulmonary hypertension, an often fatal hemodynamic abnormality that may be idiopathic, familial (or heritable), or occur secondary to other diseases such as chronic obstructive pulmonary disease (COPD).

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Pulmonary artery pressure (PAP) varies with age, however, from early childhood to the fifth decade of life, the upper limit of mean PAP is approximately 20 mmHg. PAP is a product of CO and pulmonary vascular resistance (PVR) as demonstrated by the equation: $PAP = CO \times PVR$. In this equation, PVR is the vascular resistance of the whole lung, including the pulmonary arteries, capillaries, and veins. Thus, $PAP = CO \times [PVR_{\text{arteries}} + PVR_{\text{capillaries}} + PVR_{\text{veins}}]$. According to this equation, PAP can be raised by (i) an increase in CO and/or (ii) an increase in PVR of the arteries, capillaries and veins. One would, therefore, expect a tremendous rise in PAP during periods of increased CO, such as during strenuous exercise. High intensity exercise in a healthy individual, however, only causes a slight increase in PAP due to a compensatory increase in the cross-sectional area of the pulmonary vascular bed which decreases PVR. This compensatory mechanism, which is mainly due to vasodilation (arterial distention) and recruitment of previously unperfused vessels, is dependent on the compliance of the pulmonary arterial wall. A loss or decrease of this compliance, for example, when the pulmonary vascular wall stiffens due to vascular remodeling, leads to progressively pronounced pulmonary hypertension (175, 192).

Similar to other systems and organs, the physical laws also govern the circulation of blood in the lungs. The laws of physics predict that anytime a liquid (e.g., blood) flows through a cylindrical tubular structure (e.g., a blood vessel); the resistance of such tube (i.e., PVR) is inversely proportional to the fourth power of the radius of lumen of the tube (i.e., blood vessels). This is best demonstrated by the *Poiseuille* equation:

$$PVR = 8L\eta / \pi r^4$$

where L is the length of the tube (or vessel), η is the coefficient of viscosity of blood, and r is the inner radius of the tube (or vessel). It can be seen from this equation that, in a single vessel, the resistance to blood flow is inversely proportional to the fourth power of the vessel radius (or diameter), and a very small change in the radius (or diameter) of the pulmonary vessels can significantly increase the PVR. In a vascular network of arteries, capillaries, and veins the resistance also depends on the arrangement of the network.

Consider the pulmonary arterial cast from a section of human lung shown in Figure 1A. The pulmonary arteries in this image are in both series and parallel with other arteries. Three schemes have been proposed to describe this complex branching structure: the Weibel model, the Strahler model, and the diameter-defined Strahler model. The Weibel model (Fig. 1B) assumes a symmetrical branching structure such that all arteries of the same size are in parallel (and make up an order or generation of the lung) and arteries of different sizes are in series (295). The Strahler system does not require a symmetrical branching pattern, believed to be a better physiological representation (Fig. 1C). In this scheme, one begins numbering from the smallest arteries and when two vessels of the same order meet, the order number of the parent vessel increases by one. If a small artery joins a larger artery, the order number of the larger artery does not change. This model has been applied to human and cat lungs (261, 305), but did not yield good estimates of resistance. The diameter-defined Strahler model (Fig. 1D) improves this scheme by adding a new rule stating that when a vessel of order n

with diameter D_n meets another vessel of order n , the parent vessel has order $n + 1$ if and only if its diameter is larger than $D_n + (S_n + S_{n+1})/2$ where S_n and S_{n+1} are the standard deviations of the diameters of order n and $n + 1$ (127). All three schemes have been applied to describe the pulmonary vascular networks of humans, cats, dogs, and rats (43-45, 116).

The diameter-defined Strahler system is potentially the most accurate model of the human lung vasculature and calculations based on this model best mimic *in vivo* hemodynamics. Using this scheme, investigators have identified 15 orders of pulmonary arteries between the main pulmonary artery (PA) and the capillaries, and 15 orders of pulmonary veins between the capillaries and the left atrium in healthy human lungs (116, 305). Regardless of the model used to calculate the total resistance in the pulmonary circulation, the decreased intraluminal diameter (or radius) of pulmonary arteries due to vasoconstriction, concentric vascular wall thickening and *in situ* thrombosis, as well as the decreased compliance of pulmonary arterial wall are the major causes for the elevated PVR in patients with pulmonary hypertension.

Pulmonary vasoconstriction is triggered by a rise in cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{cyt}}$) in PASMC, while sustained vasoconstriction is maintained by both Ca^{2+} -dependent and Ca^{2+} -independent mechanisms. In addition to activating myosin light chain kinase (MLCK) causing PASMC contraction and migration, an increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ also stimulates Ca^{2+} -dependent signal transduction proteins [e.g., Ca^{2+} /calmodulin-dependent protein kinase (CaMK) and mitogen-activated protein kinase (MAPK)] and transcription factors (e.g., CREB and AP-1, Ki67) to promote cell proliferation (15, 25, 110, 266, 326).

Pulmonary arterial constriction and dilatation, or PASMC contraction and relaxation, are also regulated by factors released from pulmonary arterial endothelial cells (PAEC) (Table 1). For example, release of the endothelium-derived constricting factors (EDCF), endothelin-1 (ET-1) and thromboxane A_2 (TXA_2), from PAEC causes pulmonary vasoconstriction by activating ET receptors (e.g., ET_A) and thromboxane/prostaglandin endoperoxide (TP) receptors, respectively, on the membrane of PASMC. Release of the endothelium-derived relaxing factors (EDRF), nitric oxide (NO) and prostacyclin (PGI_2), and the endothelium-derived hyperpolarizing factors (EDHF) from PAEC, however, causes pulmonary vasodilatation by stimulating synthesis of cGMP/cAMP and induces membrane hyperpolarization in PASMC. Activity (and expression) of the enzymes, for example, endothelial NO synthase (eNOS), required for synthesis and production of EDRF, EDHF, and EDCF depends, at least in part, on changes of $[\text{Ca}^{2+}]_{\text{cyt}}$ in PAEC.

A rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ in PAEC and PASMC, therefore, has differential effects on vascular tone. A rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ in PAEC, for example, causes pulmonary vasodilation by (i) increasing the activity of eNOS (producing NO), (ii) increasing the activity of cytochrome P450 (producing epoxyeicosatrienoic acids, EETs, and/or EDHFs) (91, 131), and (iii) opening Ca^{2+} -activated K^+ (K_{Ca}) channels (K^+ efflux from PAEC to the EC-SMC intercellular space, which causes membrane hyperpolarization in PASMC) (76, 232). In contrast, increased $[\text{Ca}^{2+}]_{\text{cyt}}$ in PAEC can also (i) upregulate mRNA and protein expression of mitogenic and angiogenic factors in PAEC causing pulmonary vascular remodeling (85), and (ii) increase pulmonary vascular endothelial permeability or cause pulmonary vascular barrier

dysfunction (by causing PAEC contraction), which enhances infiltration of inflammatory cells (e.g., lymphocytes, neutrophils, macrophages) and circulating progenitor cells and increases accumulation of circulating growth factors in the vascular wall to further vascular remodeling. Pulmonary vasoconstriction and vascular remodeling are both related to an increase in $[Ca^{2+}]_{cyt}$ in PASMC and PAEC.

Furthermore, Ca^{2+} sensitization of contractile apparatus in PASMC is also an important mechanism for pulmonary vasoconstriction induced by acute hypoxia (short-term hypoxia that lasts from seconds/minutes to hours) and agonists. In addition to contraction induced by a rise in cytoplasmic Ca^{2+} concentration and activated calmodulin, the contraction of smooth muscle (including pulmonary vascular smooth muscle) can be induced or maintained by a vasoconstrictor agonist without a change in $[Ca^{2+}]_{cyt}$. When a greater contraction is produced for a given level of $[Ca^{2+}]_{cyt}$, this phenomenon is usually referred to as Ca^{2+} sensitization of contractile apparatus (266). Hypoxia induces pulmonary vasoconstriction not only by increasing $[Ca^{2+}]_{cyt}$, but also by causing Ca^{2+} sensitization of contractile apparatus or an increase in the Ca^{2+} sensitivity of contractile apparatus in PASMC (126, 183, 184).

It is often difficult to standardize the definition for “acute” and “sustained” or “chronic” hypoxia, as well as for “transient” and “sustained” vasoconstriction. Usually, “acute hypoxia” is used to indicate short-term hypoxia that lasts from seconds/minutes to hours, while “chronic hypoxia” is used to indicate long-term hypoxia that lasts from days to weeks/months. “Sustained hypoxia” is usually used to indicate continuous hypoxia in contrast to “intermittent hypoxia” which is often used to indicate hypoxic exposure in a special pattern similar to changes in alveolar and/or arterial PO_2 in patients with obstructive sleep apnea. A “transient vaso-constriction” usually means that the vessel constricts at first (rapidly causing the tension to increase and reach a maximal level) and then relaxes (causing the tension to return to a low or control level) in the presence of stimulation (e.g., exposure to hypoxia or application of a vasoconstrictor). A “sustained vasoconstriction” usually indicates that the vessel constricts gradually to increase the tension to a plateau level (or sustained phase) which maintains as long as the stimulation (e.g., hypoxia or agonist) is present. “Sustained hypoxia” and “sustained vasoconstriction” are two completely different phenomena. “Sustained hypoxia” (i.e., continuous exposure to hypoxia) may cause a “transient vasoconstriction,” whereas “sustained vasoconstriction” can be caused by “acute hypoxia” (i.e., a short-term continuous exposure to hypoxia) or “chronic hypoxia” (i.e., a long-term continuous exposure to hypoxia), as well as by “sustained hypoxia” (continuous exposure to hypoxia) or “intermittent hypoxia” (exposure to hypoxia in an alternating or irregular pattern).

In intact animal and isolated perfused lung experiments, acute hypoxia (or short-term hypoxia from seconds to minutes/hours) causes a sustained increase in PAP. The sustained increase in PAP returns to the control level when the lung is ventilated with normoxic gas mixture (187, 213, 296). In isolated PA ring experiments, the observations from different laboratories are different, which may be related to the use of pretone-inducing agent. Acute hypoxia gradually increases tension in isolated rat PA rings (endothelium-intact) without precontraction using a priming factor (or a pretone-inducing agent), but not in isolated mesenteric artery (MA) ring. Removal of extracellular Ca^{2+} almost abolishes, while

verapamil significantly inhibits, the acute hypoxia-induced sustained increase in tension. Functional removal of endothelium actually enhance the acute hypoxia-induced vasoconstriction in isolated rat PA rings, indicating that the endothelium modulates (but not mediates) acute hypoxia-induced sustained pulmonary vasoconstriction (321). In isolated PA rings precontracted with a pretone-inducing agent (e.g., $\text{PGF}_{2\alpha}$), acute hypoxia often causes a two-phase increase in tension, a transient constriction (phase I) followed by a sustained vasoconstriction (phase II). Different pretone-inducing agents, however, significantly affect the kinetics of acute hypoxia-induced pulmonary vasoconstriction. For example, there is no transient phase (or phase I) vasoconstriction if 30 mM K^+ is used as a pretone-inducing agent (1). By simultaneously measuring tension and $[\text{Ca}^{2+}]_{\text{cyt}}$ in isolated PA rings, investigators have demonstrated that the phase I (transient phase) of HPV is accompanied by a transient increase in $[\text{Ca}^{2+}]_{\text{cyt}}$, whereas tension increases in the phase II of HPV but $[\text{Ca}^{2+}]_{\text{cyt}}$ remains unchanged but maintains in a higher level than control. Treatment of the isolated PA with Y-27632 (0.1–3 μM), a specific inhibitor of Rho-activated kinases (ROCK I and II), significantly attenuated both transient (phase I) and sustained (phase II) HPV. The inhibitory effect of Y-27632 on phase II vasoconstriction is, however, greater than that on phase I HPV. For example, 0.3 μM of Y-27632 decreased phase II HPV by 44% but only caused a 5% inhibition on phase I contraction, while 3 μM Y-27632 decreased phase II vasoconstriction by approximately 80% but only caused a 40% to 45% inhibition on phase I increase in tension (241, 242). These results indicate that Ca^{2+} sensitization of the contractile apparatus is involved in acute hypoxia-mediated sustained vasoconstriction (or phase II HPV).

Ion Flux and Transportation and Membrane Potential

A resting cell body behaves like a single-section resistor–capacitor (RC) circuit in response to an applied current. An electrical model or analogue of the cell membrane is thus composed of a resistor which represents the membrane resistance (R_m), a parallel capacitor which represents the membrane capacitance (C_m), and a battery which represents the resting potential (E_r) (Fig. 2A). In the electrical analogue, the membrane potential (E_m) is the total membrane potential at any time. The electrical model of membrane ion fluxes has been widely applied to excitable cells because of its predictive strength and simplicity. However, some of the assumptions based on the single-section electrical model do not apply to biological membranes. A more representative description of the changes from inactive to active membrane is provided by the parallel-conductance model (Fig. 2B). Here, the membrane is thought to contain three parallel sets of channels, one for K^+ , one for Na^+ , and one for Cl^- . Each channel is described by a battery, E (the equilibrium potential), in series with a conductance, g (equal to the inverse of the resistance). The membrane capacitance, C_m is across the whole ensemble.

Membrane permeability to cations (e.g., K^+ , Na^+ , and Ca^{2+}) and anions (e.g., Cl^- , HCO_2^+) plays an important role in regulating intracellular ion homeostasis, cell volume, and excitability. Passive flux of ions across the plasma membrane along the electrochemical gradient is controlled by the activity of ion channels, while active transportation of ions against their electrochemical gradient is regulated by the activity of ion transporters with associated energy expenditure (e.g., the catalysis of ATP).

Almost all mammalian cells, including PASMC and PAEC, maintain a negative E_m which is close to the equilibrium potential for K^+ (E_K). The cell interior is electrically negative with respect to the extracellular compartment: the electrical potential difference ranges from -85 to -60 mV in excitable cells (e.g., neurons and muscle cells) and -55 to -30 mV in nonexcitable cell (e.g., epithelial/endothelial cells, lymphocytes and neutrophils). Under resting conditions: (i) the plasma membrane is much more permeable to K^+ than to Na^+ , that is, the permeability for K^+ (P_K) is much greater than the permeability for Na^+ (P_{Na}); (ii) there is constant cation efflux through K^+ channels; and (iii) Na^+ pumps (or Na^+/K^+ ATPase) actively and constantly uptake 2 K^+ in exchange for 3 Na^+ (the Na^+ pump, by moving 3 Na^+ ions out and 2 K^+ ions in, causes a net transfer of positive charge out of the cell and thus contributes to the negative resting E_m).

The equilibrium potential for a given ion (cation or anion) is determined by the Nernst equation:

$$E_{ion} = \frac{RT}{zF} \log \frac{[X]_o}{[X]_i}$$

where E_{ion} reflects the equilibrium potential of ion X , R is the gas constant, T is the temperature (in Kelvin), z is the valence of the ion, F is the Faraday's constant and $[X]_o$ and $[X]_i$ are the concentrations of ion X in the extracellular space and in the cytosol, respectively. At body temperature (37°C), RT/zF is constant and equals approximately to 60.8 mV (when $z = 1$) ($RT/F = 25.69$ at 25°C and 26.73 at 37°C , if “ln” is used in the equation). The equation does assume that the membrane is permeable to a given ion in order for this ion to generate a transmembrane (TM) potential. For calculating the equilibrium potential for K^+ at 37°C , the Nernst equation can be simplified to: $E_K = 60.8 (\log [K^+]_o / [K^+]_i)$ or $E_K = 25 (\ln [K^+]_o / [K^+]_i)$.

A schematic illustration of the electrochemical gradients for K^+ , Na^+ , Ca^{2+} , and Cl^- that contribute to E_m as determined by data collected from PASMC is featured in Figure 2C. At resting E_m , a net cytosolic loss of K^+ and Cl^- and net gain of Na^+ (and Ca^{2+}) occurs. If the cell membrane was solely permeable to K^+ , then E_m would be directly equal to E_K . As previously mentioned, E_m in PASMC and PAEC is actually much more depolarized (than the value of E_K calculated by the Nernst equation), being in the range of -50 to -40 mV in PASMC; therefore, indicative of other ion conductances and fluxes that contribute to E_m under resting conditions. The constant-field Goldman equation quantitatively analyses the contribution of three of the ions (K^+ , Na^+ , and Cl^-) to E_m ,

$$E_m = \frac{RT}{zF} \log \frac{P_K [K]_o + P_{Na} [Na]_o + P_{Cl} [Cl]_i}{P_K [K]_i + P_{Na} [Na]_i + P_{Cl} [Cl]_o}$$

Where P_K , P_{Na} , and P_{Cl} are the absolute membrane permeability to K^+ , Na^+ , and Cl^- in cm/s, respectively, and $[X]_o$ and $[X]_i$ are the extracellular and intracellular concentrations of each ion in millimoles per liter.

According to the Goldman-Hodgkin-Katz (GHK) equation, if $P_K \gg P_{Na}$ (e.g., under resting conditions), then E_m will approach the Nernst potential for K^+ (i.e., the equilibrium potential for K^+ , E_K); $E_m \approx E_K$ when cells are under resting conditions. If $P_{Na} \gg P_K$ (when cells are electrically excited or stimulated by membrane depolarizing factors), then E_m will become close to the equilibrium potential for Na^+ (E_{Na}); $E_m \approx E_{Na}$ when action potential is generated. In summary, E_m in PASM and PAEC is a function of the Na^+ , K^+ , and Cl^- concentration gradients across the plasma membrane and the relative ion permeability (P). In resting PASM and PAEC, E_m is controlled primarily by P_K and gradient, because $P_K \gg P_{Cl} > P_{Na}$.

PASM have been shown to actively maintain an elevated cytoplasmic K^+ concentration ($[K^+]_{cyt} \approx 140$ mM) and a low cytoplasmic Na^+ concentration ($[Na^+]_{cyt} \approx 10$ mM), while resting cytoplasmic Cl^- concentration ($[Cl^-]_{cyt}$) is approximately 50 mM (Table 2). PASM have a unique resting permeability to ions; a basal permeability to Cl^- (and Na^+) helps to explain why the resting E_m (-40 to -60 mV) is substantially less negative than predicted by the equilibrium potential for K^+ ($E_K = -83$ mV).

K⁺ Channels and Transporters and Membrane Potential

The lipid bilayer of the plasma membrane is impermeable to ions (including both cations and anions). The ion permeability is directly related to the whole-cell ion current. For example, P_K is directly related to the whole-cell K^+ current (I_K) according to the following equation:

$$I_K = N \cdot i \cdot P_{open}$$

where N is the number of membrane K^+ channels, i is the single-channel current, and P_{open} is the open-state probability of a K^+ channel.

When K^+ channels close, P_K and I_K decrease and cell E_m becomes less negative or increasingly positive (i.e., the membrane depolarizes). When K^+ channels open, P_K and I_K increase and cell E_m becomes more negative close to the K^+ equilibrium potential (i.e., the membrane hyperpolarizes or repolarizes). Furthermore, when mRNA and protein expression of K^+ channels is downregulated, N (the number of K^+ channels in the plasma membrane) and I_K decrease and cell E_m becomes less negative or increasingly positive (i.e., the membrane depolarizes). When mRNA and protein expression of K^+ channels is upregulated, N and I_K increase and cell E_m becomes more negative close to the K^+ equilibrium potential (i.e., the membrane hyperpolarizes or repolarizes).

K^+ can be transported into virtually all mammalian cells against its electrochemical gradient. The mechanism responsible for this active transport is the ouabain-sensitive Na^+/K^+ pump (Na^+/K^+ -ATPase) that expels 3 Na^+ ions in exchange for 2 entering K^+ ions. Hydrolysis of the terminal high-energy phosphate bond of ATP provides sufficient energy so that the Na^+/K^+ pump can concentrate K^+ more than 20-fold in cells and thus can extrude Na^+ against an equivalent concentration gradient. This transport is electrogenic because a single

net positive charge is extruded during each cycle. The resulting current flow usually adds 1 to 2 mV to the resting E_m determined from ion gradients and permeabilities. The Na^+/K^+ pump compensates for the loss of K^+ through the various types of K^+ -permeable channels (Fig. 3A).

Based on the model described in Figure 3A, there are three major causes to induce membrane depolarization: (i) decrease in K^+ currents (I_K) due to downregulation and dysfunction of various K^+ channels [e.g., voltage-gated, Ca^{2+} -activated, and two-pore domain K^+ channels (K_{2P})]; (ii) decrease in Na^+/K^+ ATPase activity (e.g., when ouabain is increased in the plasma and extracellular fluid); and (iii) increase in Cl^- currents (I_{Cl}) due to upregulation and increased activity of Ca^{2+} -activated Cl^- (Cl_{Ca}) channels (Fig. 3B). Membrane depolarization subsequently increases $[\text{Ca}^{2+}]_{\text{cyt}}$ by inducing Ca^{2+} influx through voltage-dependent Ca^{2+} channels (VDCC) and by eliciting Ca^{2+} release or mobilization from the sarcoplasmic (SR) or endoplasmic (ER) reticulum via ryanodine receptors (RyR) (Fig. 3B). The increased $[\text{Ca}^{2+}]_{\text{cyt}}$ can be restored or recovered to the baseline level (approximately 100 nM) by (i) Ca^{2+} extrusion via the $\text{Ca}^{2+}/\text{Mg}^{2+}$ ATPase in the plasma membrane, (ii) outward Ca^{2+} transportation by the forward mode of $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX); and (iii) Ca^{2+} sequestration or uptake into the SR/ER by the $\text{Ca}^{2+}/\text{Mg}^{2+}$ pump in the SR/ER membrane [SR/ER Ca^{2+} - Mg^{2+} ATPase (SERCA)] (Fig. 3A). Inhibition of Na^+/K^+ ATPase (or Na^+ pump) and/or activation of Na^+ -permeable channels in the plasma membrane would increase $[\text{Na}^+]_{\text{cyt}}$. When $[\text{Na}^+]_{\text{cyt}}$ is increased, the forward mode of NCX would change to the reverse mode and cause inward Ca^{2+} transportation and increase $[\text{Ca}^{2+}]_{\text{cyt}}$ (Fig. 3A). In addition to causing pulmonary vasoconstriction, a rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ in PASMC is also an important stimulus for cell proliferation and migration and for gene expression.

Functional and Molecular Classification of Ion Channels

A functional channel usually comprises of the pore-forming subunit(s) and regulatory subunit(s). For example, a functional voltage-gated K^+ (K_V) channel is formed from four identical (homotetramer) and/or different (heterotetramer) pore-forming α -subunits associated with four cytoplasmic regulatory β -subunits (Fig. 4A). A VDCC is, however, formed by one pore-forming α -subunit associated with several different regulatory subunits (e.g., β -, γ -, and δ -subunits) (Fig. 4B). There are four repeats or units of the six-TM domain structure in the α -subunit, each of the units contains a pore region, which looks like the K_V channel α -subunit. These will be discussed later.

Based on the permeability or selectivity to charged ions, the channels (or the pore-forming subunits) are functionally classified into two major classes: cation channels and anion channels. Cation channels include Na^+ , Ca^{2+} , and K^+ channels, and anion channels include Cl^- channels and bicarbonate channels. This article will focus on describing the function, regulation, and pathogenic roles of various Na^+ , Ca^{2+} , K^+ , and Cl^- channels in the plasma membrane and in the membrane of intracellular organelles. The cation and anion channels can be further divided into different categories based on their biophysical and pharmacological properties (e.g., rapidly-inactivating channels vs. slowly-inactivating channels, voltage-dependent channels vs. ligand-gated channels, ATP-sensitive channels vs.

Ca²⁺-activated channels) and location (plasma membrane vs. intracellular organelle membrane). Furthermore, the ion channels and transporters can also be classified based on their molecular structure. For example, one-pore and six TM domain K⁺ channels, two-pore and four TM domain K⁺ channels or one pore and two TM domain K⁺ channels.

The ion transporters in the plasma membrane of PASMC and PAEC can be grouped into two categories: (i) the transporters that require energy released from catalysis of ATP to transport ions against their electrochemical gradient, such as Na⁺/K⁺ ATPase (or Na⁺ pump), Ca²⁺/Mg²⁺ ATPase (or Ca²⁺ pump); and (ii) the transporters that transport or exchange ions based on the ion concentration gradient (ATP is not required for the ion transportation, but is involved in regulating the activity of the transporters), such as NCX (30).

Ca²⁺ Channels and Transporters

As mentioned earlier, [Ca²⁺]_{cyt} in PASMC is critical for the regulation of pulmonary arterial constriction and vascular tone. During homeostasis, [Ca²⁺]_{cyt} is maintained at ~100 nM creating a gradient greater than 10,000 fold with the extracellular environment (~1.8 mM) (111). There are several fundamental systems coordinating to control [Ca²⁺]_{cyt}: (i) Ca²⁺ entry (CEE) through VDCC, receptor-operated Ca²⁺ channels (ROC) and store-operated Ca²⁺ channels (SOC); (ii) Ca²⁺ release from the SR/ER via Ca²⁺ release channels, for example, ryanodine receptors (RyR) and IP₃ receptors (IP₃R) on the SR/ER membrane; (iii) Ca²⁺ sequestration into the SR/ER via the SR/ER Ca²⁺-Mg²⁺ ATPase (SERCA); (iv) Ca²⁺ extrusion via Ca²⁺-Mg²⁺ ATPase in the plasma membrane; (v) outward and inward Ca²⁺ transportation, respectively, through the forward and reverse mode of NCX in the plasma membrane (328); and (vi) Ca²⁺ release from, and sequestration into, lysosome and mitochondria (also see Fig. 3A).

Role of intracellular Ca²⁺ in PASMC contraction and proliferation

Intracellular Ca²⁺ signals are precisely regulated to coordinate (i) muscle contraction (skeletal, cardiac, and smooth muscle) and cell motility; (ii) neurotransmitter release, neuronal excitability, and learning and memory; (iii) fertilization and development; (iv) cell proliferation, differentiation, and apoptosis; and (v) gene transcription (26, 27). The contractile characteristics and the mechanisms that cause contraction of vascular smooth muscle (including pulmonary vascular smooth muscle) are very different from cardiac muscle and skeletal muscle. PASMC undergo slow, sustained, and tonic contractions. The major contractile apparatus (or contractile proteins) in PASMC are actin and myosin, which are not organized into distinct bands as they are in cardiac and skeletal muscle cells, but are organized in such a way as to maintain the sustained contractions.

Contraction in PASMC can be initiated by two major mechanisms: (i) electromechanical coupling, whereby contraction or relaxation is initiated by changes in E_m ; and (ii) pharmacomechanical coupling, consisting of a series of complex signalling pathways that are not dependent on changes in E_m . Regardless of the initial mechanisms, an increase in [Ca²⁺]_{cyt} is the major trigger for smooth muscle cell contraction and pulmonary vasoconstriction. When [Ca²⁺]_{cyt} rises, Ca²⁺ binds to calmodulin (CaM) and forms Ca²⁺/CaM complex, which activates MLCK to phosphorylate the myosin light chain. This

phosphorylation increases the activity of myosin ATPase that hydrolyzes ATP, thereby releasing energy. The subsequent cycling of the myosin cross-bridges (actomyosin) produces displacement of the myosin filament in relation to the actin filament causing contraction. The phosphorylated myosin light chain can be de-phosphorylated by Ca^{2+} -independent myosin light chain phosphatase (MLCP), leading to smooth muscle cell relaxation (Fig. 5).

Activation of RhoA/Rho kinase (ROK), via GPCR activation and Ca^{2+} /CaM, can also cause smooth muscle contraction by inactivating MLCP and causing Ca^{2+} sensitization in pulmonary arterial smooth muscle. ROK can directly phosphorylate and inactivate MLCP causing smooth muscle contraction. ROK can also indirectly inhibit MLCP and cause Ca^{2+} sensitization of contractile proteins by activating the MLCP-inhibitor protein (CPI-17) (203).

Cytosolic Ca^{2+} also stimulates cell proliferation by activating Ca^{2+} -sensitive signal transduction proteins (e.g., CaMK, MAPK) and transcription factors (e.g., NFAT, CREB, c-Fos/c-Jun, and NF- κ B) (Fig. 6). Since the nuclear envelope has high permeability to Ca^{2+} , a rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ can rapidly increase nuclear $[\text{Ca}^{2+}]$ and activate Ca^{2+} -sensitive pathways in the nucleus. Maintaining a sufficient level of Ca^{2+} in the SR/ER is also critical for cell proliferation; SERCA is the major active transporter that sequesters cytosolic Ca^{2+} into the SR/ER and maintain a high $[\text{Ca}^{2+}]$ (~0.1-0.5 mM) in the SR/ER ($[\text{Ca}^{2+}]_{\text{SR}}$). $[\text{Ca}^{2+}]_{\text{SR}}$ may regulate cell proliferation by modulating the amplitude and frequency of Ca^{2+} signals in the cytosol and nucleus.

Ca^{2+} also participates in regulating PASMC proliferation through its intimate involvement with the cell cycle. There are four Ca^{2+} /CaM-sensitive steps in the cell cycle: (i) transition from G_0 (resting state) to G_1 phase (the beginning of DNA synthesis); (ii) from G_1 to S phase (an inter-phase during which replication of the nuclear DNA occurs); (iii) from G_2 to M phase (mitosis); and (iv) through M phase (63,186). A rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ in PASMC, which also rapidly increases nuclear $[\text{Ca}^{2+}]$ and gradually enhances stored $[\text{Ca}^{2+}]$ in the SR/ER, plays an important role in facilitating quiescent cells to get into the cell cycle and propelling cell passage through the cell cycle. Therefore, an increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ not only induces pulmonary vasoconstriction by triggering PASMC contraction, but also mediates pulmonary vascular remodeling by stimulating cell proliferation and migration (Fig. 6).

Regulation of $[\text{Ca}^{2+}]_{\text{cyt}}$ in PASMC

Voltage-dependent Ca^{2+} channels—Membrane depolarization-mediated increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ is caused by opening of VDCC in the plasma membrane. VDCC is activated when membrane is depolarized to a threshold, usually, at the range of -40 to -30 mV. Like other voltage-dependent channels, VDCC can also be inactivated by membrane depolarization; therefore, the total Ca^{2+} influx through VDCC during membrane depolarization is dependent on the window current. Only a few millivolts are necessary to open VDCC and allow Ca^{2+} influx, causing contraction (40, 90).

VDCC can be functionally classified into six different types (L, T, N, P, Q, and R types) based on functional characteristics (e.g., single-channel conductance, voltage dependence, time-dependent kinetics of channel opening and closing, pharmacological properties, and cell/tissue distributions (280). The L-type channels (L for “long-lasting”) are high voltage

activated and sensitive to 1,4-dihydropyridine agonists (e.g., Bay K 8644). L-type channels are ubiquitously expressed in excitable cells and many nonexcitable cells, and are major pathways for voltage-gated CCE involved in muscle contraction (including skeletal, cardiac and smooth muscles). The T-type channels (T for “transient”) are low voltage activated and rapidly inactivating VDCC that are resistant to dihydropyridine antagonists. The N-type channels (N for neural/non-L) are also high voltage-activated channels but they are significantly different from L-type channels. The N-type channels are resistant to dihydropyridines and largely blocked by ω -conotoxin; these channels are largely restricted to neurons. The currents through P-type channels (P for “Purkinje”) are relatively high voltage activated and sustained currents that are not sensitive to either dihydropyridines or ω -conotoxin, but sensitive to ω -agatoxins. The Q-type channels are similar to the P-type channels that are high voltage activated and resistant to dihydropyridines and ω -conotoxin, but sensitive to ω -agatoxin. The Q-type channels are mainly distributed in cerebellar granule cells. The R-type channels (R for “residual/resistant”) are intermediate voltage activated and resistant to all dihydropyridines, ω -conotoxin, and ω -agatoxin (53, 280). In vascular smooth muscle cells (including PASM), L- and T-type channels are the major pathways for voltage-mediated CCE involved in excitation-contraction coupling (90, 275) and cell proliferation (143).

The pore-forming $\alpha 1$ -subunit of VDCC comprises of four linked segments each containing six TM domains (S1-S6) and is enclosed by N- and C- termini both located in the intracellular site. The Ca^{2+} selective pore in the $\alpha 1$ -subunit is between S5 and S6, and the voltage sensor is located in S4. Many regulatory subunits (e.g., β_{1-4} , γ_{1-8} , and $\alpha 2\delta_{1-3}$) can co-assemble with the $\alpha 1$ -subunits giving rise to heterogeneity of functional VDCC (Fig. 4B). Sites for channel regulation by intracellular second messengers, toxins, and drugs also reside in the $\alpha 1$ -subunit. The pore-forming $\alpha 1$ -subunit is commonly associated with an intracellular β -subunit and a disulfide-linked TM $\alpha 2\delta$ -subunit.

There are multiple genes that have been demonstrated to encode the pore forming α -subunits, such as $\text{Ca}_v1.1$ (α_{1S}), $\text{Ca}_v1.2$ (α_{1C}), $\text{Ca}_v1.3$ (α_{1D}), $\text{Ca}_v1.4$ (α_{1F}), $\text{Ca}_v2.1$ (α_{1A}), $\text{Ca}_v2.2$ (α_{1B}), $\text{Ca}_v2.3$ (α_{1E}), $\text{Ca}_v3.1$ (α_{1G}), $\text{Ca}_v3.2$ (α_{1H}), and $\text{Ca}_v3.3$ (α_{1I}). Different $\alpha 1$ -subunits and the combination of $\alpha 1$ -subunits with the accessory subunits form six functionally distinct subfamilies: the L-, N-, P-, Q-, R-, and T-type channels (54). Table 3 provides a summary of the expression and pharmacological regulation of VDCC in the pulmonary vasculature. The high voltage-activated and slowly inactivating L-type channels are highly expressed in vascular smooth muscle cells, while the low voltage-activated and rapidly inactivating T-type channels have also been shown to be present in PASM. The L-type VDCC seems to be not functionally expressed in PAEC based on RT-PCR, Western blot, and electrophysiological experiments. However, lung vascular endothelial cells functionally express high level of T-type channels (299).

L-type (Ca_v1) channels have been substantially characterized in PASM and are important in elevating $[\text{Ca}^{2+}]_{\text{cyt}}$ during hypoxia. Furthermore, differences in Ca^{2+} channel density and oxygen sensitivity have been observed along the PA tree (92). L-type channels are high voltage activated and have a 25-pS single channel conductance ($\text{Ca}_v1.2b$ in the lung). A high affinity binding site for inhibitors such as the dihydropyridines (nifedipine), the

phenylalkylamines (verapamil) and the benzothiazepines (diltiazem) resides in the TM $\alpha 1$ -subunits close to the pore region (113). The electrophysiological characteristics of VDCC in PASMC include an activation threshold between -40 and -50 mV with the maximum inward current amplitude occurring at approximately 0 mV (204). Inhibition of L-type channels in PASMC attenuates voltage-gated Ca^{2+} influx and improves pulmonary arterial pressure in $>40\%$ of infants with PAH (18) and 10% to 15% of adult patients with PAH (237). L-type channels are shown to be upregulated in hypoxia-induced pulmonary hypertension (112). Although the L-type VDCC is critical in membrane depolarization-mediated Ca^{2+} influx during hypoxia, it is not the only source for the increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ during hypoxic pulmonary vaso-constriction (HPV), as pharmacological inhibition of L-type channels only partially abolishes HPV (19, 281).

The other common Ca^{2+} channel in the pulmonary vas-culature is the low voltage-activated T-type (Ca_v3). Its single channel conductance of 8 pS is insensitive to the common L-type channel blockers. R-type ($\text{Ca}_v2.3$) currents have been shown to be activated by ET-1 (28) and cause enhanced cerebral artery constriction during subarachnoid hemorrhage (122, 158). Recent studies have also suggested that R-type channels are important in the proliferation of human PASMC (243).

Receptor-operated and store-operated Ca^{2+} channels—When membrane receptors, G protein-coupled receptors (GPCR) or tyrosine kinase receptors (TKR), in the plasma membrane are activated by extracellular ligands, phospholipase C (PLC) is activated to stimulate synthesis of IP_3 and diacylglycerol (DAG), two important second messengers. DAG can open ROC, leading to Ca^{2+} influx and increase in $[\text{Ca}^{2+}]_{\text{cyt}}$. IP_3 can activate IP_3 receptors (IP_3R) on the SR/ER membrane, stimulate Ca^{2+} release or mobilization from the SR/ER to the cytosol, and ultimately deplete Ca^{2+} in the SR/ER. Depletion of Ca^{2+} from the SR/ER triggers store-operated Ca^{2+} entry (SOCE), or capacitative CCE (64, 231), by activating SOC. The precise molecular entity of ROC and SOC has remained enigmatic. In the vasculature or vascular smooth muscle and endothelial cells, there is increasing evidence that transient receptor potential channels (TRPC) are involved in forming functional ROC and SOC channels.

In addition, Ca^{2+} release-activated Ca^{2+} channels (CRAC) have been identified as a functional CCE pathway in several cell types. ROC, SOC, and CRAC are probably not mutually exclusive in vascular smooth muscle and endothelial cells. Recently, studies provide new evidence demonstrating a coexistence and interaction between the subunits forming CRAC [e.g., Orai and stromal interaction molecule 1 (STIM-1)] and the TRPC forming SOC in a variety of cell types including smooth muscle cells (46, 219, 290).

Transient receptor potential channels: TRPC are part of the superfamily of six TM spanning cation channels with the N- and C-termini both located in the cytoplasm; however, they lack the voltage-sensing moiety. They function as voltage-independent, nonselective cation channels, being variably permeable to Na^+ , K^+ , Cs^+ , Li^+ , and Mg^{2+} , in addition to the predominantly carried ion Ca^{2+} (211). Several subtypes of TRPC have been identified in many cell types: the canonical TRP (TRPC1-7), vanilloid receptor-related TRP (TRPV1-4) and melastatin-related TRP (TRPM1-8). Other TRP subfamilies, which tend to be associated

with specific genetic disorders, are the polycystins (TRPP), mucolipidins (TRPML), the ankyrins (TRPA), and the mechanoreceptor potential C (TRPN) (217). Over 10 TRP isoforms are expressed in the vasculature. Expression of mRNA, protein, or both of TRPC1-7, TRPV1-4, TRPP2, and TRPM2-4, -7, -8 has been implicated in different vascular smooth muscle and endothelial cells.

In PASMC and PAEC, more than 10 TRP isoforms have been detected; the function and expression of these channels are summarized in Table 4 and the membrane topology and representative currents are depicted in Figure 7. Distinct domains in the N- and C-termini reflect the capability to form specific protein-protein interactions; some are ubiquitous, others isoform specific. All TRP isoforms, for example, have a series of ankyrin repeats; however, the number of repeats may differ among isoforms. Other functionally recognized domains include calmodulin-binding sites, present in TRPC1 and TRPC4, acting to regulate Ca^{2+} -dependent feedback inhibition of SOCE via TRPC1 (259). TRPC1, which is ubiquitously expressed in the vasculature, is one of the pore-forming subunits comprising SOC in vascular smooth muscle cells (including PASMC and PAEC) (22, 300). Furthermore, TRPC1 is a critical TRP isoform responsible for pulmonary vasoconstriction and PASMC proliferation induced by SOCE (146, 271). In addition to forming homotetrameric channels, TRPC1 is also reported to form heterotetrameric channels with TRPC5, TRPC3, and TRPP2; such interaction may be crucial to the trafficking or translocation of the TRPC1 to the plasma membrane (71, 114). TRPC4 is widely expressed and known to be present in vascular smooth muscle cells (327), however, its role is more pertinent in the endothelium where it regulates vascular permeability and vasorelaxation (93, 277). TRPC1, 4 and 6 protein expression varies with location in the pulmonary vascular tree, higher expression was observed in the distal PA than in the proximal, this correlated with changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ occurring during HPV (20). Furthermore, TRPM 2-4 and 7-8 and TRPV 1-4 have been detected using RT-PCR in the PA (88, 119, 279).

Ca^{2+} release-activated Ca^{2+} channels: Recent evidence suggests a significant role for CRAC in voltage-independent Ca^{2+} influx into cells and it is postulated that this may contribute to SOC in PASMC (290). Orai-1 is the fundamental pore-forming subunit for CRAC in the plasma membrane (46,219, 306). Ca^{2+} concentration in the SR/ER is sensed by STIM-1 and, upon store depletion, it oligomerizes and enabling it to redistribute to discrete sites in the SR/ER membrane in close proximity to the plasma membrane (166).

Both STIM-1 and Orai-1 are highly expressed in vascular smooth muscle cells including mesenteric artery smooth muscle cells (MASMC) and PASMC (164, 165, 291) and in PAEC (62). Inhibition of STIM1 and STIM2 with siRNA significantly attenuates SOCE in PASMC isolated from distal pulmonary arteries of rats (164). These data indicate that Orai and STIM are functionally expressed in PASMC and PAEC, and may play an important role in SOCE or CCE.

Cellular mechanisms involved in SOCE: Role of STIM and Orai: Through genome-wide RNAi screening, investigators have identified the Ca^{2+} sensor that detects levels of Ca^{2+} in the SR/ER as STIM1. STIM1 is a 685 amino acid long, single TM protein that is expressed on the SR/ER membrane and plasma membrane (161, 162). Near the N-terminus of STIM1

is an EF-hand domain that senses $[Ca^{2+}]_{SR/ER}$ (which is in the range of 0.5-2 mM). When Ca^{2+} is not bound to the EF-hand domain, for example, under the condition when the SR/ER Ca^{2+} store is depleted, STIM1 undergoes a conformational change, which allows it to multimerize and translocate to the SR/ER-plasma membrane junction (or puncta), bind with Orai1 tetramers and/or TRPC subunits on the plasma membrane, activates SOC, and induce SOCE (Fig. 8) (46,244). Mutagenesis of the EF-hand domain (mimicking low Ca^{2+}) leads to STIM1 multimerizing in distinct regions of the plasma membrane, called puncta, in the absence of store depletion and to activating SOC (159, 160, 166). Examination by the total internal reflection fluorescence (TIRF) microscopy shows that store depletion-induced puncta, which contains STIM1, localizes 10-20 nm from the plasma membrane allowing for SR/ER STIM1 to interact with SOC in the plasma membrane (159, 160).

There are two isoforms in the STIM family, STIM1 and STIM2, which share a high sequence similarity (<65%) and analogous sequence-based domain architectures. However, the kinetics of STIM oligomerization, which is an important step to induce SOCE, is different between STIM1 and STIM2 (267, 268). For example, the kinetic half-time of conversion from a monomeric state to a steady-oligomeric state is more than 70 times shorter for STIM1 EF-hand and sterile α -motif domains than STIM2 under similar conditions. Urea-induced rates of unfolding for STIM1 EF-hand and sterile α -motif domains are more than three times faster than STIM2. These data indicate that distinct oligomerization dynamics of STIM1 and STIM2 may explain why these two structurally similar isoforms differentially affect SOCE in various cells. STIM1 may be involved in the fast and transient phase of SOCE, while STIM2 may be responsible for the sustained Ca^{2+} influx through SOC/CRAC.

Plasmalemmal Ca^{2+} -ATPase—The Ca^{2+} - Mg^{2+} ATPase in the plasma membrane (PMCA) is a plasmalemmal P-type Ca^{2+} -ATPase regulated by calmodulin which extrudes Ca^{2+} from cells in PASMC and PAEC to maintain Ca^{2+} homeostasis. It is encoded by four genes (PMCA 1-4) of which the vascular smooth muscle expresses PMCA-4 and, to a lesser extent, the PMCA-1 (212). Alternative splicing of these four genes has created 20 isoforms of the PMCA. Each PMCA is a 10-TM spanning domain with cytosolic N- and C- termini. The calmodulin-binding site is located in a key regulatory domain in the C-termini along with protein kinase C (PKC) and A (PKA) phosphorylation sites (103). Interestingly, its activity has been shown to increase in the presence of oxidants, such as hydrogen peroxide, and matrix metalloprotease 2 (MMP-2) in PASMC (174). The pump has recently been purified from bovine PASMC, which will open the door for more detailed studies on its ion transport mechanisms (173) and potential role in the development of pulmonary vascular diseases.

Na^+/Ca^{2+} exchanger—The NCX in its forward mode represents the second system functioning to export Ca^{2+} from intact cells. The NCX has a much larger capacity than the PMCA and its importance as a primary mechanism for Ca^{2+} extrusion has been well established. The expression and function of the NCX has been widely documented in both normal and diseased PASMC and PAEC (31, 251, 324, 330). Mammalian cells maintain a low cytoplasmic concentration of Na^+ ($[Na^+]_{cyt}$, ~10 mM) compared to the extracellular

concentration of Na^+ ($[\text{Na}^+]_{\text{out}} \sim 140 \text{ mM}$) because of the activity of the Na^+ - K^+ ATPase. The TM Na^+ gradient can be utilized to energize the NCX, which moves Na^+ and Ca^{2+} across the membrane in the opposite direction. Two families of plasma membrane NCX proteins have been described in mammalian tissues, one in which Ca^{2+} movement depends only on Na^+ (NCX family), and the other in which Ca^{2+} movement depends on Na^+ and K^+ (NCKX family). The stoichiometry of NCX is 3 Na^+ per 1 Ca^{2+} , while NCKX is 4 Na^+ per 1 Ca^{2+} and 1 K^+ . Both NCX and NCKX can operate either in a forward (Ca^{2+} exit) or reverse (CCE) mode, depending on the Na^+ , Ca^{2+} (and K^+) gradients and membrane potential (E_m). For a constant extracellular $[\text{Ca}^{2+}]$ (1.8-2 mM) and $[\text{Na}^+]$ ($\sim 140 \text{ mM}$), $[\text{Ca}^{2+}]_{\text{cyt}}$ is a cubic function of $[\text{Na}^+]_{\text{cyt}}$ and an exponential function of E_m . Therefore, a small change in $[\text{Na}^+]_{\text{cyt}}$ or E_m can cause a large change in $[\text{Ca}^{2+}]_{\text{cyt}}$ (30, 31).

Many functional studies have demonstrated that the plasma membrane NCX is involved in regulating Ca^{2+} homeostasis of blood vessels. The functional evidence for the sarcolemmal NCX in vessels is also supported by direct demonstration that the NCX is expressed in vascular smooth muscle and endothelial cells. There are three currently identified isoforms of NCX, NCX1-3, and a further four isoforms for NCKX (30). The isoforms have a similar overall structure consisting of an intracellular loop, which connects to a five-TM domain at the N-terminal and a six-TM domain at the C-terminal. Protein and mRNA for the NCX1 and NCKX3 isoforms have been detected from rat PA and cultured human PASM. Removal of extracellular Na^+ , which alters the TM Na^+ gradient and activates the reverse mode of NCX, significantly increases $[\text{Ca}^{2+}]_{\text{cyt}}$ in PASM due to the inward transportation of Ca^{2+} . This indicates that the NCX could contribute to CCE due to NCX activity in the reverse mode (inhibited by KB-R9756). Indeed, the protein and mRNA expression of NCX1, an NCX family member of NCX proteins, is upregulated in PASM from patients with idiopathic pulmonary arterial hypertension (IPAH) compared with PASM from normal subjects or normotensive patients. By activating the reverse mode of NCX, removal of extracellular Na^+ caused a rapid increase in $[\text{Ca}^{2+}]_{\text{cyt}}$, which is significantly enhanced in IPAH PASM compared with normal PASM. Furthermore, passive depletion of intracellular Ca^{2+} stores using cyclopiazonic acid (10 μM) not only causes a rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ due to Ca^{2+} influx through SOC, but also mediated a rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ via the reverse mode of NCX. The upregulated NCX1 in IPAH PASM lead to an enhanced CCE via the reverse mode of NCX, but did not accelerate Ca^{2+} extrusion via the forward mode of NCX. These observations indicate that the upregulated NCX1 and enhanced CCE via the reverse mode of NCX are an additional mechanism responsible for the elevated $[\text{Ca}^{2+}]_{\text{cyt}}$ in PASM from IPAH patients. It is therefore predicted that blockade of the reverse mode NCX may be a possible therapeutic target in the treatment of pulmonary hypertension (324, 328).

Functional interaction of TRPC and NCX in caveolae

Caveolae are cholesterol- and sphingolipid-rich flask-shaped invaginations of the plasma membrane, whose principal structural component is caveolin. In the vasculature, caveolae appear to be more prominent in endothelial cells than in smooth muscle cells. However, the number of caveolae and expression level of caveolin is significantly increased in PASM from patients with IPAH (Fig. 9A). The “simplest” physiological role of caveolae and caveolin is

to concentrate and colocalize cooperative receptors, signaling molecules/proteins, and effectors (e.g., ion channels and transporters) within microdomains.

A caveolin-scaffolding domain (CSD) on caveolin itself can associate with membrane proteins. The TRPC1 proteins contain a C-terminal CSD-consensus binding sequence that allows for their physical and functional interaction with caveolin-1 (Cav-1) in the caveolae of PASM. Cav-1 also binds to an N-terminal cav1-binding motif (CBM; aa 322-349) of TRPC1 (Fig. 9B). Truncation of cav-1 (i.e., removal of CSD, membrane anchoring domain, and palmitoylation sites) significantly decreases SOCE through TRPC and downregulates TRPC1 and cav-1 expression.

The presence of TRPC and NCX proteins in these caveolar lipid raft domains constitutes an important functional factor in regulating intracellular Ca^{2+} . TRPC-mediated Na^+ influx into the cytosol of PASM, such as that provoked by activation of GPCR (Fig. 9C) and depletion of Ca^{2+} in the SR/ER, is one of the major mechanisms to increase cytosolic $[\text{Na}^+]_{\text{cyt}}$ ($[\text{Na}^+]_{\text{cyt}}$). A rise in $[\text{Na}^+]_{\text{cyt}}$ (especially in the sub-membrane area) functionally converts the forward (inward Na^+ and outward Ca^{2+} transportation) mode of NCX to the reverse (outward Na^+ and inward Ca^{2+} transportation) model. The inward Ca^{2+} transportation through the reverse model of NCX plays an important role in maintaining a high level of $[\text{Ca}^{2+}]_{\text{cyt}}$ and refilling Ca^{2+} into the SR/ER via SERCA (Fig. 9C).

By binding with cav-1 in both N- and C-termini, TRPC can be efficiently regulated upon activation of receptors. As shown in Figure 9C, accumulated ligand (e.g., growth factors, mitogenic and angiogenic ligands, and vasoactive substances) in caveolae activates the receptors (GPCR or RTK) located in caveolae and increases synthesis of IP_3 and DAG. By binding with IP_3 receptors (IP_3R) on the ER/SR membrane, the second messenger IP_3 induces a transient increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ due to Ca^{2+} release from the SR/ER to the cytosol. Opening of the Ca^{2+} release channels or IP_3R is an efficient way to deplete Ca^{2+} from the intracellular stores (i.e., SR or ER). Such store depletion then activates TRPC-encoded SOC, located in caveolae, and promote Ca^{2+} influx or SOCE. The proximity of the receptor, the channel, the transporter, and caveolin allows the ligand-mediated increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ to be efficiently and rapidly regulated for signal transduction and for stimulating functional changes of the cell.

K⁺ Channels and Transporters

Activity of K^+ channels and Na^+ pump in the plasma membrane is the key regulator of E_m , and plays an important role in the regulation of vascular contractility and smooth muscle excitability. Increased efflux or outward current of K^+ after activation of K^+ channels leads to membrane hyperpolarization, inhibition of VDCC and vasodilation. A small change in K^+ channel activity can have a substantial effect on membrane potential and thus factors targeting K^+ channels can modulate vessel tone (66). The main families of K^+ channels identified in the pulmonary vasculature are; (i) K_v channels, (ii) large-, intermediate-, and small-conductance Ca^{2+} -activated K^+ channels (BK_{Ca} , IK_{Ca} , and SK_{Ca} , respectively), (iii) ATP-sensitive K^+ channels (K_{ATP}), and (iv) K_2p . The Na^+ - K^+ ATPase is the chief

mechanism for Na^+ extrusion, actively transporting both Na^+ and K^+ against their concentration gradients.

Voltage-gated K^+ channels

K_v channels in PASMC carry a rapidly inactivating A-type current, a slowly inactivating delayed rectifier current, and a noninactivating delayed rectifier current. Most of these currents are activated in the voltage range of resting E_m in PASMC (except the A-type current); the unitary conductance of K_v channels in PASMC is 5 to 65 pS. 4-Aminopyridine (4-AP) is a potent blocker of K_v channels. Functionally, the noninactivating or slowly inactivating delayed rectifier K_v channels are the major determinants of resting E_m and, thus, $[\text{Ca}^{2+}]_{\text{cyt}}$ and tonic tension. Inhibition of K_v channels in PASMC (e.g., by 4-AP or hypoxia) depolarizes the cells, induces Ca^{2+} -dependent action potentials, raises $[\text{Ca}^{2+}]_{\text{cyt}}$, and causes vasoconstriction. In contrast, activation of K_v channels in PASMC by agents like nitric oxide hyperpolarizes PASMC, closes VDCC, and causes pulmonary vasodilation.

K_v channels are the most diverse group of K^+ channels ubiquitously expressed in vascular smooth muscle cells (59, 222). There are currently approximately 40 K_v channel members grouped into 12 subfamilies (109) among which there are more than 20 K_v channels that are expressed in the pulmonary vasculature (Table 5). Functional K_v channels are homo- and/or hetero-tetramers composed of four pore-forming α -subunits and four regulatory β -subunits. The pore-forming α -subunit protein contains an N-terminus in the cytosol, six TM domains [(S1-S6), a pore region located between S5 and S6], a voltage sensor located in S4 (and S5), and a C-terminus in the cytosol. The central pore is highly selective to the transportation of K^+ across the cell membrane (Figs. 4A and 10A) (168). The voltage sensor contains a preserved region of positive Arg or Lys amino acid residues (repeats of Arg or Lys-Xaa-Xaa₍₇₎) essential for depolarization-dependent opening of the channel (5). Selectivity to K^+ is energetically favored through a flexible pore lined by a backbone of carbonyl groups, a sequence highly conserved within the K^+ channel family (201). Although the cytoplasmic C-terminus differs between K_v channel α -subunits, the cytoplasmic N-terminus highly conserved throughout the K_v channel family. This 120-amino acid N-terminal domain contains a tetramerization domain (T1) comprising of the molecular determinants for the assembly of functional tetrameric channels from specific α -subunits and the association of the homo/heterotetrameric α -subunits with the regulatory β -subunits (147). Furthermore, the tetrameric T1 domain exists as a region distinct to the TM channel and, due to a structural resemblance, is commonly referred to as a ball and chain structure (104). The highly conserved N-terminal T1 domain of K_v channels is important for proper channel assembly, gating, inactivation kinetics, and association with regulatory β -subunits (253). Recently, two non-synonymous mutations (G182R and E211D) in the *KCNA5* gene, located in the T1 domain, have been identified from patients with IPAH (42). Experiments *in vitro* indicate that the mutation (or polymorphism) at nt. 182, which lead to the amino acid change from glycine to argine (G182R), significantly alters the inactivation kinetics of *KCNA5* (or $\text{K}_v1.5$) channels and markedly affects the channel protein localization onto the plasma membrane. The amino acid residue E211 may be involved in the interaction of *KCNA5* channels with regulatory proteins. These data support a role for the highly-conserved T1 domain in channel kinetics as well as in *KCNA5* channel subcellular localization.

Activity of K_v channels is a key in the regulation of resting E_m and membrane repolarization in PASMC (11, 14, 208, 264, 317, 318). Whole-cell currents carried by K_v channels, denoted as $I_{K(V)}$, are determined by the following equation:

$$I_{K(V)} = N \times P_{\text{open}} \times i_{K(V)}$$

where N denotes the total number of K_v channels, P_{open} is the steady-state open probability of a K_v channel, and $i_{K(V)}$ is the current amplitude through a single K_v channel. Decreased $I_{K(V)}$ may occur due to: (i) a decrease in the total channel protein due to transcriptional inhibition of the K_v channel genes via transcriptional silencer elements [e.g., the $K_v1.5$ repressor element (KRE) in the promoter region of the gene] (190,191), (ii) a decrease in the functional surface expression of K_v channels due to inhibition of channel trafficking by the K_v channel interacting proteins (e.g., KChIP) (153) or due to the inhibition of α - α -subunit assembly via the cytoplasmic N-terminal T1 domain (147), (iii) association with the regulatory β -subunits either decreasing activation and/or increasing inactivation of K_v channels (3, 4, 214, 215), (iv) inhibition of channel activity by PKC-mediated phosphorylation of the channel α - and β -subunits (298), (v) pharmacological blockade of the K_v channels by selective (e.g., 4-AP, bepridil, and correolide) (41, 135) and nonselective (e.g., nicotine, ET-1, serotonin, fenfluramine, acute hypoxia, and dichloroacetate) inhibitors (185,220, 221,223, 228,235), (vi) redox regulation of K_v channel function (in which oxidizing agent activates K_v channels, whereas reducing agent inhibits K_v channel activity) (11, 12, 322), and (vii) transcriptional/translational and functional inhibition of K_v channels by anti-apoptotic proteins (e.g., Bcl-2) (77).

In PASMC, heterogeneity of $I_{K(V)}$ exists (133, 225). Different $I_{K(V)}$ were recorded in PASMC isolated from conduit pulmonary arteries. Sixty-seven percent of the cell population comprised a large, rapidly activating $I_{K(V)}$ inhibited by 4-AP but insensitive to tetraethylammonium (TEA) (termed I_{Kv1}) and 33% of the cells expressed a smaller, slower activating current which was more sensitive to TEA than 4-AP (termed I_{Kv2}). Resistance pulmonary arteries exhibited a third more uniform current resembling that of I_{Kv1} , but distinguished by a higher current density, a significantly larger time constant for activation and more negative $I_{K(V)}$ activation and inactivation (263).

In addition to the K_v channels described above, PASMC also functionally express KCNQ channels, previously known as members of the K_v7 family of K_v channels. KCNQ channels were first identified in cardiac cells where mutations in a chromosomal loci on the KVLQT1 gene encode for an inherited form of long QT syndrome (LQT) (283). Five isoforms (KCNQ1-5) have been identified and, of these, only the KCNQ1 is known to be expressed in PASMC (129). Linopiridine and XE991, specific blockers of KCNQ channels, were demonstrated to be potent and selective constrictors of pulmonary arteries. Electrophysiological and molecular biological studies also demonstrate that KCNQ channels could contribute to the regulation of resting E_m in PASMC (129). Inhibition of KCNQ channels and other K_v channels (e.g., KCNA and KCNB channels) has been demonstrated to cause sustained membrane depolarization in animal and human PASMC. These observations

imply that hypoxia inhibits multiple K_v channels to ensure its depolarizing effect on PASMC and constrictive effect on pulmonary arteries. Experiments *in vivo* and *in vitro* both show that (i) acute hypoxia causes sustained pulmonary vasoconstriction in intact animals and isolated perfused/ventilated lungs, and (ii) acute hypoxia causes a two-phase pulmonary vasoconstriction in isolated pulmonary arteries consisting of a transient contraction (phase I) followed by a sustained constriction (phase II) (241) (also see discussion on page 4). Hypoxia may initially decrease the background K^+ currents generated by K^+ efflux through K_{2P} channels, KCNQ channels, and KCNA/KCNB channels to initiate membrane depolarization. Hypoxia-mediated inhibition of K_{Ca} channels (as well as the delay-rectifier K_v channels) inhibits membrane repolarization and helps maintain the membrane depolarization and causes sustained pulmonary vasoconstriction. Indeed, *in vitro* experiments demonstrate that hypoxia decreases whole-cell and single-channel K^+ currents through KCNA/KCNB channels, KCNQ channels, K_{2P} channels, and KCa channels in rat and human PASMC (106, 130, 206, 207, 230, 320). How hypoxia affects different K^+ channels, and whether hypoxia directly or indirectly affects the channel function will be discussed later.

Ca²⁺-activated K⁺ channels

K_{Ca} channels are activated by an increase in $[Ca^{2+}]_{cyt}$ and membrane depolarization. There are three types of K_{Ca} channels: small conductance (4-14 pS; SK channels), intermediate conductance (100-200 pS; IK channels), and large conductance (200-285 pS; BK or maxi-K channels). Apamin blocks SK channels, but negligibly affects BK and IK channels. BK channels are very sensitive to charybdotoxin, iberiotoxin (IBTX) and low dose of TEA (<1 mM). Voltage and Ca^{2+} gating for K_{Ca} channels are synergistic; therefore, K_{Ca} channels play a critical role in coupling changes in $[Ca^{2+}]_{cyt}$ to changes in E_m . K_{Ca} channels are half-maximally activated between +12 and +30 mV, and most are closed in vascular smooth muscle cells under resting conditions, where $E_m = -60$ to -40 mV and $[Ca^{2+}]_{cyt} = 50$ to 100 nM. Toxins that inhibit K_{Ca} channels enhance membrane depolarization and elevate $[Ca^{2+}]_{cyt}$ in stimulated vascular smooth muscle cells. Normally, K_{Ca} channels control E_m and vascular tone by negative-feedback regulation of the degree of membrane depolarization caused by myogenic factors and vasoactive substances.

Like K_v channels, functional BK_{Ca} channels are tetramers consisting of 4 pore-forming α -subunits associated with four regulatory β -subunits (Fig. 10B). The α -subunit of the BK channel is encoded by the slowpoke gene (*Slo*) and the channel protein shares extensive homology with the K_v channels of the *Shaker* (K_v1) subfamily. The SK channel has a membrane topology similar to those of K_v and BK channel α -subunits. The $\beta 1$ -subunit of the BK channel tunes the Ca^{2+} sensitivity and voltage dependence of the channel. Deletion of the gene that encodes this subunit reduces the Ca^{2+} sensitivity of BK channels at given membrane potentials. BK_{Ca} channels are ubiquitously expressed in vascular smooth muscle cells and can be activated by both membrane depolarization and $[Ca^{2+}]_{cyt}$ increase (8, 137, 163). The α -subunit has seven TM domains and features extracellular N- and intracellular C-termini (282). The channel is regulated by the β -subunit which comprises two membrane-spanning domains separated by an extracellular loop. Inhibition of Ca^{2+} influx decreased the

BK_{Ca} current and increased the $I_{K(V)}$, indicating that $[Ca^{2+}]_{cyt}$ is a critical regulator of these channels (67).

As mentioned earlier, hypoxia inhibits BK_{Ca} channels in rat and human PASMC, the hypoxia-mediated decrease in large-amplitude BK_{Ca} currents may contribute to maintaining sustained membrane depolarization during hypoxia. A recent study, however, shows that acute hypoxia-induced pulmonary vasoconstriction is comparable in wild-type mice and BK_{Ca} channel knock-out mice (BK $\alpha^{-/-}$). The amplitude and kinetics of both transient and sustained phases of hypoxia-induced pulmonary vasoconstriction are not significantly changed in isolated perfused lungs from BK $\alpha^{-/-}$ mice or hemogenase-2 knock-out mice (HO-2 $^{-/-}$). These data indicate that, although BK_{Ca} channels are regulated by HO-2-mediated redox changes, the HO-2/BK_{Ca} complex may not serve as an oxygen sensor in acute hypoxia-induced pulmonary vasoconstriction (218, 245).

Inward rectifier K⁺ channels

Rectifiers conduct current in one direction only. Inward rectifier K⁺ (K_{IR}) channels conduct inward K⁺ current but little outward current; they are blocked by intracellular Mg²⁺ and Cs⁺ and external Ba²⁺. K_{IR} channels in PASMC and PAEC are involved in setting the resting E_m , preventing membrane hyperpolarization by the electrogenic Na⁺, K⁺-ATPase, mediating K⁺-induced vasodilation, and minimizing loss of intracellular K⁺. In contrast to K_V and K_{Ca} channels, K_{IR} channels contain only two TM domains (M1 and M2). Heteromultimeric combinations of different K_{IR} channel subunits form distinct K⁺ channels; for example, the acetylcholine-sensitive, G protein-gated K⁺ channel is a heteromultimer composed of two distinct types of K_{IR} subunits, K_{IR}3.1, and K_{IR}3.4.

ATP-sensitive K⁺ channels

Native K_{ATP} channels are voltage-dependent channels that have low open probability under basal conditions. K_{ATP} channels are inhibited by intracellular ATP and glibenclamide, and activated by intracellular adenosine diphosphate (ADP) and cromakalim. They fall into two categories: low-conductance (10-50 pS) and large-conductance (=130 pS) K_{ATP} channels. In coronary and cerebral arteries, metabolic regulation of basal tone and blood flow involves modulation of K_{ATP} channels. During hypoxia or ischemia, ATP falls and ADP rises. This activates K_{ATP} channels, hyperpolarizes smooth muscle cells, and contributes to systemic vasodilation.

Functional K_{ATP} channels consist of an octameric complex of four pore-forming subunits (K_{IR} channel subunits, e.g., K_{IR}6.1 or K_{IR}6.2) and four sulphonylurea receptors (SURs) (Fig. 10C) (6). K_{IR}6.2/K_{IR}6.1 also serves as the ATP sensor, whereas SUR1 confers sensitivity to sulphonylureas (e.g., diazoxide) and ADP. Co-expression of SUR subunit 2B (SUR2B) with K_{IR}6.1 or K_{IR}6.2 produces channels with characteristics of two distinct K_{ATP} families, K_{NDP} (named reflecting a primary role for various nucleotide diphosphates in their activation) and K_{ATP} (predominantly sensitive to ATP), respectively (23). The expression of K_{IR}6.1 with SUR2B has been detected in human PASMC (68). Some evidence suggests that they may contribute to regulating resting E_m in PASMC, although the regulatory mechanisms are still to be elucidated (68). Additionally, K_{ATP} channels appear not to play a

role in HPV because that K_{ATP} channels are normally closed in the pulmonary arteries and are not activated by the levels of hypoxia that cause pulmonary vasoconstriction (240).

Two-pore domain K^+ channels

The pore-forming subunit of K_{2P} channels contains four TM domains (denoted M1-M4), two pore-forming regions (P1 and P2), a short cytoplasmic N-terminus, and a long cytoplasmic C-terminus. In addition, an extracellular loop exists between the M1 and P1 regions (as shown in Fig. 10D) (151). It is believed that two α -subunits of K_{2P} are necessary to form functionally active channels. K_{2P} are expressed in PASMC and have a noninactivating current, denoted I_{KN} , characterized by a lack of both voltage and time dependency (108). To date, more than 14 K_{2P} channels have been identified and these are subcategorized into five structural groups: (i) TWIK-1 (KCNK1), TWIK-2 (KCNK6), and KCNK7; (ii) TWIK-related acid-sensitive K^+ channel (TASK-1) (KCNK3), TASK-3 (KCNK9), and TASK-5 (KCNK15); (iii) TREK-1 (KCNK2), TREK-2 (KCNK10), and TRAAK (KCNK4); (iv) TASK-2 (KCNK5), TALK-1 (KCNK16), and TALK-2 (KCNK17); and (v) THIK-1 (KCNK13) and THIK-2 (KCNK12).

Of these channels, TASK-1, TASK-2, TREK-1, TREK-2, and TWIK-2 have been detected in the pulmonary circulation (95, 107, 136, 216). K_{2P} channels are considered proponents of the regulation of resting E_m because the outward currents through K_{2P} channels are considered “background” K^+ currents that are required for setting and maintaining the resting E_m . TASK-1 (107), in particular, is able to regulate E_m by 10 mV in both a hyperpolarizing and depolarizing direction (207). In PASMC from rats and rabbits (108) and, most recently from humans (207), the pharmacological profile of I_{KN} has been shown to match TASK-1, being insensitive to glibenclamide, TEA, and Ca^{2+} channel blockers and having a high sensitivity to external pH (but not $[Ca^{2+}]_{cyt}$). TASK-1 currents display outward rectification under physiological conditions and TASK-1 is a pure K^+ -selective leak channel (as shown in Figure 10D) (75). Inhibition of this channel by acid (H^+) is dependent upon membrane voltage and $[K^+]$, indicative of a pore-blocking inhibition mechanism. It has been shown that TASK-1 senses changes in pH within a physiological range via protonation of a histidine residue in the P1 pore-forming domain which leads to block of the channel (202). It is also noteworthy that TASK-1, in addition to its acid sensitivity, exhibits sensitivity to hypoxia. As such, TASK channels are proposed to be functionally important in mechanisms underlying HPV (108). There is currently limited evidence suggesting a direct role of TASK in HPV or PAH; however, it has recently been shown that ET-1, a vasoconstrictive agonist and mitogenic peptide known to be upregulated in PAH, inhibits TASK-1 in human PASMC (274).

Na^+ - K^+ ATPase

More than 25% of ATP produced by our body is used by Na^+ - K^+ ATPase, or Na^+ pump, to maintain a high intracellular K^+ concentration and a stable TM K^+ and Na^+ gradient, as well as a negative membrane potential, in various cells. The Na^+ pump enables the active transportation of Na^+ and K^+ . In each electrogenic “cycle,” the pump transports three Na^+ outside of cell in exchange for two K^+ into cell; the net change in charge elicits a negative

potential inside the cell close to the equilibrium potential for K^+ . Activity of the Na^+-K^+ ATPase is important to maintain a high cytosolic $[K^+]$ (140 mM) and a low cytosolic $[Na^+]$ (approximately 10-12 mM).

At molecular level, the pump is a heterodimer formed from a catalytic α -subunit and a glycosylated β -subunit (138). The α -subunit is functionally important, conferring the binding sites for the physiological ligands (namely Na^+ , K^+ , Mg^{2+} , and ATP) and for ouabain, an inhibitor that displays tissue specific affinity for the pump. The β -subunit functions as a chaperone that ensures correct insertion of the α -subunit in the plasma membrane and regulates the affinity of the enzyme for cations. There are several distinct isoforms of the α -subunit; the α_1 isoform is ubiquitously expressed, whereas the α_2 - α_4 isoforms are more limited in their distribution. It has recently been shown that the $\alpha_2\beta_1$ and $\alpha_1\beta_1$ isoform of Na^+/K^+ -ATPase exist in small membrane microdomains known as caveolae in PASM, whereas only the $\alpha_1\beta_1$ iso-form is detected in the non-caveolae regions of the plasma membrane (98).

The Na^+-K^+ ATPase utilizes energy derived from ATP hydrolysis to undergo conformational changes to enable binding and release of Na^+ and K^+ (262). The pump (the α -subunit) is also regulated by phosphorylation via PKAPKC (29) and by small membrane proteins with a characteristic FXYD motif (97). Regulation of the Na^+-K^+ ATPase expression and function has a crucial role in the regulation of the contractility of the vasculature (55). In PA, hypoxia inhibits the Na^+-K^+ ATPase (273), which would cause membrane depolarization, open VDCC, increase $[Ca^{2+}]_{cyt}$, and cause vasoconstriction.

Sodium Channels and Transporters

Voltage-dependent Na^+ channels

A rapidly activating and inactivating inward Na^+ current (I_{in}) was first described in rabbit PASM by Okabe *et al.* in 1988 (205). The current has since been further characterized in cultured human PASM (226). Although the presence of voltage-gated Na^+ channels (or currents) is certain, their functional role in the PA is not clear because the resting membrane potential in PASM (approximately -40 mV) is much less negative than that of which the voltage-gated Na^+ channels would be completely inactivated.

The rapidly inactivating voltage-gated Na^+ current in PASM is sensitive to tetrodotoxin (TTX), a specific Na^+ channel inhibitor; however, extracellular application of TTX has no effect on the resting membrane potential, and does not significantly affect Ca^{2+} release or PASM proliferation (226). It is still unclear whether Na^+ influx through voltage-gated Na^+ channels is one of the mechanisms to increase cytosolic $[Na^+]$, which can subsequently change the forward mode of NCX to the reverse mode, and enhance the inward transportation of Ca^{2+} via NCX.

Na^+ channels consist of four internally homologous sub-units each containing six TM domains, the S5-S6 TM domains fold to create an internal pore (Fig. 11A). These subunits form a single principle pore region: the structural basis of this pore determines the selectivity and conductance properties of the channel (176). There are currently over 10 voltage-

dependent Na⁺ channel genes (SCN1A-11A) identified; of these, it is predominantly those forming TTX-insensitive channels (Na_v1.5) have been identified at the mRNA expression level in cultured PASMCM (Table 6). A TTX-sensitive current has, however, been recorded in rabbit PASMCM, characterized by a very rapid inactivation (<10 ms) (61). In cultured human PASMCM, mRNA was detected for Na⁺ channel genes, SCN2A, 3A, 4A, 7A, 8A, 9A, and 11A and accessory β -subunits SCN1B and 2B (Table 6) (176). The rapid inward current in cultured human PASMCM was sensitive to sub-micromolar concentrations of TTX (128, 176). Figure 11 (B and C) shows representative Na⁺ currents recorded from cultured human PASMCM superfused with Ca²⁺-free solution. Extracellular application of 1 μ M TTX abolishes the I_{in} indicating that these are TTX-sensitive voltage-gated Na⁺ currents.

Epithelial Na⁺ channels

The amiloride-sensitive epithelial Na⁺ channel (ENaC) is a voltage-independent and Na⁺-selective channel that plays an important role in Na⁺ and water transport in the lung (and other organs, such as kidney and colon) and is most frequently associated with defective Cl⁻ transport in patients with cystic fibrosis. ENaC in airway epithelial cells is also involved in high-altitude pulmonary edema (HAPE) (155, 248, 269). During HAPE, hypoxia inhibits reabsorption of Na⁺ and concomitant fluid from the alveolar space by inhibiting ENaC in the apical membrane and Na⁺/K⁺-ATPase in the basolateral membrane of alveolar epithelial cells. In animal models, inhibition of ENaC with RNA interference significantly inhibits fluid absorption *in vivo* in rat lungs (155), while in humans, activation of ENaC with β -adrenergic agonist (e.g., salmeterol) decreases the incidence of HAPE in susceptible subjects by more than 50% (248).

In renal glomerular vasculature, ENaC has been demonstrated to function as a mechanoreceptor that regulates myogenic constriction or myogenic vascular tone. Using a *Caenorhabditis elegans* (*C. elegans*) mechanotransducer model, investigators demonstrated that ENaC proteins form the ion-conducting pore of the mechanotransducer. In vascular smooth muscle cells, functional ENaC channels, which are formed by β -ENaC and γ -ENaC subunits, are expressed on the surface membrane and co-localized with smooth muscle α -actin (72, 73, 124, 125). The lack of α -ENaC in vascular smooth muscle cells implies that the functional ENaC channels in blood vessels (formed by β -ENaC and γ -ENaC subunits) may be different from the “classical” ENaC channels in pulmonary and renal epithelial tissues (mainly formed by α -ENaC subunits). The channels formed by β -ENaC and γ -ENaC subunits in vascular smooth muscle cells can be mechanically activated. For example, pressure- or stretch-gated Na⁺ and Ca²⁺ influx and vasoconstriction in isolated mouse renal interlobar arteries and in rat juxtamedullary afferent arterioles are inhibited by amiloride (5 μ M)- and benzamil (1 μ M)-specific ENaC inhibitors (102, 124). Thus, the importance of ENaC in the myogenic constrictor response is not limited to the renal interlobar artery, which may also play a critical role in myogenic constriction, as well as smooth muscle cell migration, in other vascular beds. Whether ENaC is functionally expressed in the pulmonary vasculature and how ENaC is involved in the regulation of pulmonary vascular tone and pulmonary vascular remodeling remain unclear and need further studies.

Sodium-dependent exchangers

In addition to the $\text{Na}^+\text{-K}^+$ pump that requires ATP and energy to transport Na^+ and K^+ against their TM electrochemical gradients, there are several Na^+ -dependent transporters or exchangers that do not require ATP to function, but use the potential energy generated by the TM ion gradients to transport these ions. For example, the TM Na^+ gradient is the major driving force for the function of NCX (as discussed earlier in this article). Two other Na^+ -dependent exchangers are expressed and functional in the pulmonary circulation as well; the $\text{Na}^+\text{-H}^+$ exchanger (NHE) and the $\text{Na}^+\text{-Mg}^{2+}$ exchanger (NME). Such extrusion mechanisms are functionally important in the maintenance of ion homeostasis and the regulation of cell functions.

$\text{Na}^+\text{/H}^+$ exchanger—The NHE is electrogenic, exchanging Na^+ for H^+ and is primarily involved in pH homeostasis in many mammalian cell types (intracellular pH is approximately at 7.2 under physiological conditions), acting alongside several other ion transport systems such as the Na^+ -independent $\text{Cl}^-/\text{HCO}_3^-$ and Na^+ -dependent $\text{Cl}^-/\text{HCO}_3^-$ transporters. The NHE is activated by an increase in intracellular H^+ concentration and extracellular Na^+ is exchanged for intracellular H^+ in a 1:1 stoichiometry. Excessive stimulation of the exchanger will ultimately increase intracellular Na^+ , which, in turn, can drive the $\text{Na}^+\text{-K}^+$ ATPase increasing the energy demands of the cell and/or activate the NCX-driving Ca^{2+} into the cell (through the reverse mode of NCX).

The NHE has recently been studied in PASM; the observations indicate that it plays an important role in cell apoptosis and proliferation and in hypoxia-induced pulmonary hypertension. Chronic hypoxia (for 3 weeks) significantly increases intracellular pH and upregulates the NHE mRNA expression in rat PASM, which may subsequently contribute to the increased PASM proliferation and migration in hypertrophied pulmonary arteries. Inhibition of the NHE by 5-(N,N-dimethyl)amiloride (DMA), however, enhances the rate of apoptosis in rat PASM (304). Similar results are obtained from mouse PASM as well. Chronic exposure of mouse PASM to hypoxia raises intracellular pH, increases NHE activity, and upregulates NHE1 expression (238); such changes have been confirmed to contribute to the development of hypoxia-induced pulmonary hypertension. Furthermore, hypoxia-mediated pulmonary vascular remodeling and pulmonary hypertension are both inhibited in mice with deficiency of NHE1 (309). In addition, the NHE is also involved in recovery from acid loads in pulmonary vascular smooth muscle cells during hypoxia (169).

There are nine isoforms of the NHE (NHE1-9) (247). NHE2-9 are more restricted in their distribution, unlike NHE1, which is ubiquitously expressed in the plasma membrane. The structure of the 815 amino acid NHE protein is similar among different isoforms; NHE1 shares 42% amino acid homology with NHE2, 38% with NHE3, and 43% with NHE4; NHE2 shares 36% homology with NHE3 and 38% with NHE4; and NHE3 shares 39% homology with NHE4. The NHE contains 10-12 helical hydrophobic membrane spanning domains (M1-10), an ~500 amino acid N-terminus functionally responsible for catalyzing the amiloride-sensitive NHE and an ~300 amino acid C-terminal acting as a regulatory domain. Serines of cytoplasmic C-terminus are phosphorylated at resting and stimulated

states, while serines or threonines of extracellular domains are (*N*-linked and *O*-linked) glycosylated (200).

Na⁺-Mg²⁺ exchanger—The NME has been widely studied in erythrocytes and in vascular smooth muscle cells with a pertinent role in systemic hypertension identified; however, is yet to be cloned and extensively studied in the pulmonary vasculature. The NME presents a predominant mechanism for Mg²⁺ extrusion from the cell and has been identified in a variety of tissues including: sheep ruminal epithelial cells (250), vascular smooth muscle cells (278), and erythrocytes (239). Interestingly, activation of the NME has been shown to modulate Na⁺-Mg²⁺ transport in vascular smooth muscle cells and increased activity of NME could influence the change in Na⁺-dependent regulation of intracellular [Mg²⁺] observed in spontaneously hypertensive rats (SHR) (278).

Intracellular Mg²⁺ is an important cation required for enzyme activity. Cytosolic Mg²⁺ has been demonstrated to directly regulate different ion channels in different cell types. An important role for intracellular Mg²⁺ in the regulation of K_v channels in rat PASMC has recently been shown and presents a potential for the intracellular [Mg²⁺] and activity of the NME to contribute to the development of HPV and pulmonary arterial hypertension (PAH) (89). Cytosolic [Mg²⁺] or trans-SR/ER membrane Mg²⁺ gradient (between the SR/ER and cytosol) is also involved in regulating activity of the SR/ER Ca²⁺-Mg²⁺ ATPase (SERCA). Accumulated Mg²⁺ in the cytosol or decreased [Mg²⁺] in the SR/ER would affect Ca²⁺ sequestration from the cytosol to the SR/ER, indirectly change intracellular Ca²⁺ homeostasis, and ultimately have an effect on pulmonary vasoconstriction and vascular remodeling.

Chloride Channels and Transporters

Chloride ion (Cl⁻) is the most abundant intracellular and extracellular anion in many cell types. Using different methods, investigators have revealed that intracellular or cytosolic [Cl⁻] in vascular smooth muscle cells is very high (51-86 mM) (see Table 2), which corresponds to a reversal potential for Cl⁻ in the range of -25 to -12 mV. Therefore, activation of Cl⁻ channels under resting conditions in PASMC (where *E_m* is approximately -40 to -50 mV) results in Cl⁻ efflux (or inward current), which would cause membrane depolarization.

Cl⁻ can be actively accumulated in the cell via one of three uptake mechanisms: the Na⁺-K⁺-2Cl⁻ co-transporter, the Cl⁻/HCO₃ exchanger and/or the relatively unknown “pump III” (60). In pulmonary vascular smooth muscle and endothelial cells, the most extensively studied Cl⁻ channel current is stimulated by an elevation of [Ca²⁺]_{cyt}, commonly referred to as the CL_{Ca} current (*I*_{CLCa}). In addition, voltage-gated and volume-sensitive Cl⁻ channels exist, though are not as well characterized in the pulmonary vascular system (Table 7). Cl⁻ transportation is involved in the regulation of cell volume including apoptotic volume decrease (AVD), and implicated in essential cellular functions such as progression of the cell cycle as well as cell proliferation, migration, contraction, and excitability. Alterations of Cl⁻ transportation would thus have a pertinent role in the development of pulmonary edema and potentially pulmonary vascular remodeling.

Ca²⁺-activated Cl⁻ channels

Cl_{Ca} channels are stimulated by an elevation of [Ca²⁺]_{cyt}, either Ca²⁺ release or mobilization or Ca²⁺ influx. In rat and rabbit PASM, receptor-mediated increase in [Ca²⁺]_{cyt} (e.g., by norepinephrine and histamine) increases *I*_{ClCa} (115, 288). Furthermore, increased [Ca²⁺]_{cyt} due to inward transportation of Ca²⁺ via the reverse mode of NCX can also increase *I*_{ClCa} in PASM (150). In primary cultured PASM from rats, membrane depolarization-mediated increase in [Ca²⁺]_{cyt} elicits a time-dependent outward Cl⁻ current (due to Cl⁻ influx) that reverses at a potential close to the Cl⁻ equilibrium potential and is sensitive to Ca²⁺ influx. Replacement of extracellular Ca²⁺ with Ba²⁺, which enhances the inward cation (Ba²⁺) currents through L-type VDCC, abolishes the outward Cl⁻ currents (due to Cl⁻ influx) and inward tail currents (due to Cl⁻ efflux) (319). These observations indicate that the Cl⁻ currents are obviously induced by Ca²⁺ influx (Figs. 12A and 12B). Furthermore, these currents are also sensitive to niflumic acid, a Cl⁻ channel blocker. Extracellular application of 10 to 50 μM niflumic acid significantly reduced the outward Cl⁻ currents and inward tail currents (Fig. 12C). Inhibition of the Cl_{Ca} channels with niflumic acid also attenuates agonist-mediated membrane depolarization in PASM and vasoconstriction in isolated pulmonary arterial rings. These observations indicate that activity of Cl⁻ channels (or amplitude of *I*_{ClCa}) in PASM is involved in regulating pulmonary vascular tone by directly regulating *E*_m and indirectly modulating Ca²⁺ influx through VDCC. While the electrophysiological and pharmacological properties of *I*_{ClCa} are relatively well defined, the molecular correlate for *I*_{ClCa} in the PA is not precisely known.

Based on the biophysical and pharmacological properties, the Cl_{Ca} channels can be divided into three distinct families, (i) channels activated directly by Ca²⁺, (ii) channels requiring CaMKII, and (iii) cGMP-dependent channels. A group of proteins, known as CL_{Ca}, which have been implied to associate with *I*_{ClCa}, are the bestrophin family (182). Expression of bestrophin proteins (*Best-1-4*) in different cell types correlates the appearance of a Cl⁻ conductance. The cGMP-dependent *I*_{ClCa} was recently characterized in several vascular beds, including the PA; a high [Ca²⁺]_{cyt} sensitivity, a linear voltage dependence, and permeability to anions (182, 270). Using siRNA, *Best-3* was shown to be a critical component of the endogenous *I*_{ClCa} in smooth muscle cells of intact PA walls (182). While *Best-3* may encode a “non-classical” Cl_{Ca} channel due to the lack of voltage sensitivity, it is the “classical” Cl_{Ca} channel that is expressed at its highest level in the PA. In a recent breakthrough study, Caputo and colleagues provided evidence that the membrane protein TMEM16A is an intrinsic component of the voltage-sensitive Cl_{Ca} channel and its expression in the pulmonary vasculature remains to be investigated (51). TMEM16A is an 840 amino acid, 8 TM-spanning protein with cytoplasmic N- and C-termini; it undergoes alternative splicing generating a variety of isoforms with significant sequence homology (e.g., TMEM16b, ~60% sequence homology). The TMEM16 proteins may represent subsets of Cl⁻ channels with biophysical profile that remains to be characterized.

Elevation of [Ca²⁺]_{cyt} also activates an outwardly rectifying Cl⁻ current in PAEC for which the reversal potential is dependent on extracellular [Cl⁻]. The current inactivates rapidly at negative membrane potentials and has a single-channel conductance of approximately 8 pS. Furthermore, the currents are significantly inhibited by chelation of intracellular Ca²⁺ with

1,2-bis(o-aminophenoxy)ethane- N,N,N',N' -tetraacetic acid (BAPTA) and by extracellular application of 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) and niflumic acid (199). These data indicate that Cl_{Ca} channels are also functionally expressed in PAEC, the agonist-mediated increase in $[Ca^{2+}]_{cyt}$ can activate Cl_{Ca} channels, cause membrane depolarization, decreases the driving force for Ca^{2+} influx through voltage-independent Ca^{2+} channels (e.g., TRPC and nonselective cation channels), and ultimately decrease $[Ca^{2+}]_{cyt}$ (and EDHF/NO production).

In addition to its role in the regulation of E_m and ion transportation in PASMC and PAEC, Cl_{Ca} channels are also involved in cell adhesion by interaction with integrins (e.g., integrin β_4) (2). The transportation-independent function of Cl_{Ca} channels for cell adhesion may contribute to regulating pulmonary vascular permeability. These observations indicate that cell junction, especially among endothelial cells, is not only controlled by traditional adhesion molecules and proteins, but also regulated by ion channels and transporters including the Cl_{Ca} channel, Na^+/K^+ ATPase (α_3 - and β_2 -subunit), voltage-gated Na^+ channel β -subunit, VDCC ($\alpha_{2\delta 1}$ -subunit), $K_v 2.1$ channel, ionotropic glutamate receptor (δ_2 -subunit), and water channel [aquaporin (AQP)] (142). The precise mechanisms involved in regulating pulmonary vascular endothelial cell junction via these ion channels and transporters remain unclear. The transport-independent function, along with the transport-dependent function, may significantly contribute to the regulation of vascular permeability, vascular tone, and vascular remodeling.

Voltage-gated Cl^- channels

The voltage-gated Cl^- channels are thought to be encoded by the family of chloride channel (CLCN) genes. There are nine members in the family of CLCN channel subunits which share significant similarity in sequence homology, but there is a distinct diversity in function among different CLCN channels. CLCN1 to 7, CLCNKa, and CLCNKb are the currently identified voltage-sensitive Cl^- channels. The CLCN channel characteristically has 10 TM spanning domains with intracellular C- and N-termini and it is suggested that functional channels arise from dimerization of CLCN proteins. The expression of CLCN3 has been shown in the human fetal lung and in PASMC and PAEC isolated from the large and small pulmonary arteries, and in pulmonary epithelial and bronchial smooth muscle cells (148). The first functional evidence demonstrated an upregulation of the CLCN3 gene in both rat PA in the monocrotaline-induced model of pulmonary hypertension and PASMC cultured from canines incubated with inflammatory mediators. Furthermore, overexpression of CLCN3 improved cell viability and increased resistance of these cells to reactive oxygen species (69), potential role in the pathogenesis of pulmonary hypertension.

Volume-sensitive Cl^- channels

Volume-sensitive Cl^- channels are ubiquitously expressed in many cell types, which can be activated in response to cell swelling. Functional volume-sensitive Cl^- channels are present in canine PASMC (301). Exposure of cells to hypotonic solutions increases Cl^- conductance and elicits a current exhibiting outward rectification in a symmetrical Cl^- gradient. The volume-sensitive Cl^- channel can be inhibited by DIDS, ATP, and tamoxifen. It is currently

unknown, however, whether this current is carried by a homomeric channel or CLCN3 in heteromeric association (301).

Aquaporins

Aquaporins are membrane bound proteins that form heterotetramers in the cell membrane and facilitate the transporter of water and, in some cases, other small solutes (e.g., glycerol) across the membrane. There are currently 13 mammalian homologues in the AQP family that have been detected in different tissue and cell types; several of these are functionally expressed in the human lung. AQPs are six-TM domain-spanning proteins with five interconnecting loops and intra-cellular N- and C-termini. Two hydrophobic loops, the cytoplasmic B loop and the extracellular E loop each contain a signature motif consisting of a tripeptide sequence asparagine, proline, and alanine, are proposed to form the aqueous pore region of the channel (94). The water pores, however, are impermeable to charged species (e.g., H^+ or rather hydronium ions, H_3O^+), an important property that is critical for maintaining electrochemical potential across the membrane. Each AQP monomer contains two hemipores, which fold together to form the pore of the water channel.

AQPs are important for water permeability across micro vessels in intact lungs; more specifically, using knockout mice, AQP1 was demonstrated to provide an important route for osmotic and hydrostatic water movement between the airspace, interstitial compartment and micro vessels (16, 52). AQPs are most influential in regulating pulmonary fluid homeostasis and, in a bleomycin-induced pulmonary edema rat model; the expression of AQP1 has been shown to be significantly reduced. It is postulated that alterations in AQP expression may lead to pathophysiological conditions (16, 123).

Several AQP transcripts have been identified in human and rat PASMC using RT-PCR. Function of AQP water channels in pulmonary vascular smooth muscle and endothelial cells is involved in the regulation of cell volume and AVD. Recently, the water channels or AQPs have also been demonstrated to transport gas (e.g., O_2 and CO_2); the “pore” for the flow of water and gas is different in the AQP-formed heterotetrameric channels (196).

Gap Junction and Gap Junction Channels

Gap junction-mediated intercellular communication

Smooth muscle and endothelium in both systemic and pulmonary arteries are, in someway, both detectors and effectors in the regulation of vascular function. The activation of either cell type (PASMC or PAEC) leads to a coordinated response in the other. When a single cell or a small number of cells is stimulated, the response can be propagated to adjacent cells producing a coordinated effect on a segment of the vessel. The coordinated intercellular communication in blood vessels is mainly due to the existence of gap junctions (GJ). Similar to systemic vascular smooth muscle and endothelial cells, PASMC and PAEC are functionally connected by GJs, which allow a direct transfer of electric signals (e.g., action potentials, depolarized, or hyperpolarized E_m) and signaling molecules (i.e., Ca^{2+} , IP_3 , DAG, and cAMP) from cell to cell. GJ are mainly formed by hydrophilic channels that bridge the opposing membrane of neighboring cells and play an important role in

intercellular communication, both along and across the vessel axis (i.e., from PASMC to PASMC, from PASMC to PAEC, and from PAEC to PASMC) (Fig. 13).

There are two general classes of communication that are known to occur in the vessels: (i) longitudinal electrical conduction that coordinates membrane potential changes along a segment of a pulmonary blood vessel and produces more uniform vasoconstrictive or vasodilative responses and (ii) membrane potential changes, originated from one PASMC (or a small group of cells) and propagated longitudinally to other PASMC, which modulate the activity of VDCC on the surface membrane and the SR/ER membrane (e.g., RyR) to cause Ca^{2+} influx and/or Ca^{2+} release, and coordinates vasomotor tone (or vasoconstriction and vasodilatation) over vessel segments several millimeters in length. Such coordination may be crucial in minimizing flow heterogeneity in the microcirculation (86, 252) and in minimizing turbulent flow and shear stress in the pulmonary circulation. Longitudinal transportation of intracellular Ca^{2+} , along with the signaling molecules that induce Ca^{2+} influx (e.g., DAG) and release (e.g., IP_3), is an important mechanism that causes a uniformly coordinated vasoactive response in pulmonary arteries and capillaries (188, 307).

Longitudinal signaling is complemented by radical movement of current or signaling molecules (e.g., Ca^{2+} , IP_3) between the two cell types via GJs located at the smooth muscle-endothelial cell interface, that is, myoendothelial junctions (MEJ) (121, 249) (Fig. 13). The tight coupling between the smooth muscle and endothelium at the MEJ allows a rise in Ca^{2+} in one cell to trigger complementary or opposing signaling events in the other cell type. As mentioned earlier, for example, a rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ in PAEC would cause pulmonary vasodilation by increasing eNOS activity and production of NO, while an increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in PASMC causes pulmonary vasoconstriction. Such functional coupling between PASMC and PAEC may activate compensatory processes; stimulation of PASMC by a vasoconstrictor increases $[\text{Ca}^{2+}]_{\text{cyt}}$ causing contraction, while the increased $[\text{Ca}^{2+}]_{\text{cyt}}$ in PASMC can drive Ca^{2+} through the MEJ to PAEC activating Ca^{2+} -dependent eNOS that produces NO, and antagonizes or minimizes the magnitude of the smooth muscle contraction. Dysfunction of the MEJ has been shown to play an important (or pathogenic) role in vascular dysfunction in many cardiopulmonary and metabolic diseases (9, 172).

PASMC and PAEC both express functional VDCC (112, 294) and K_{Ca} channels (17, 21). These channels provide opportunity for interactions between the smooth muscle and endothelium, for example, when changes in PASMC membrane potential are coupled to PAEC through the MEJ, gating of VDCC in PAEC can modulate activity of both K_{Ca} channels and endothelial NO synthesis (eNOS activity is Ca^{2+} -dependent). The main Ca^{2+} influx pathway in PAEC, however, is by voltage-independent Ca^{2+} channels, such as ROC and SOC that are potentially formed by TRPC, Orai, and STIM (39, 165, 260, 279, 302).

Endothelium-dependent hyperpolarization (EDH) of smooth muscle cells is a process commonly attributed to a freely diffusible, EDHF (96). However, EDH can also be caused by a simple transmission of an electrical signal from PAEC to PASMC via GJs in the MEJ (56-58, 120). The change in membrane potential in endothelial cells has been attributed to activation of small (SK) and intermediate (IK) conductance

K_{Ca} channels. Activation of SK/IK and membrane hyperpolarization in PAEC, which increases the driving force for Ca²⁺ influx through voltage-independent Ca²⁺ channels (e.g., TRPC) and increases eNOS-mediated NO production, may “directly” cause membrane hyperpolarization in PASM, close VDCC, and inhibit PASM contraction and pulmonary vasoconstriction.

Gap junction channels: role of connexin

In mammals, GJ channels are composed of two hemichannels, termed connexons, each provided by one of the two neighboring cells. Although a “gap” is left between the adjacent cell membrane, two connexons interact and dock on the extracellular space to form a tightly sealed, double-membrane intercellular channel (Fig. 14A). Structural analyses have shown that each connexon is composed of six polytopic TM protein subunits, termed connexins (Cx). Cx are encoded by a large gene family predicted to comprise at least 20 isoforms in humans (101, 145, 265). All represent structurally conserved non-glycosylated TM proteins (25-62 kDa in size) that differ mainly in the length of their cytoplasmic C-terminal domain. Of the 20 isoforms, Cx32, Cx37, Cx40, Cx43, and Cx45 have been identified in vascular smooth muscle and endothelial cells (83). In PASM and PAEC, at least Cx37 (197), Cx40, and Cx43 have been identified using immunocytochemistry as well as immunoconfocal and immunoelectron microscopy (134).

The topological model for Cx indicates that the polypeptide traverses the lipid bilayer four times (the four TM domains are termed M1-M4) with both the N- and C-termini facing the cytoplasm. Analysis of the different Cx indicates that M3 TM domain has an amphipathic character, suggesting that it contributes to the lining of the channel. The two extracellular loops (E1 and E2) are thought to be involved in initiating the interaction between connexons in adjacent cells. A set of three cysteine residues exists in each of the extracellular loops with a characteristic arrangement that is a signature of Cx (Fig. 14B).

Interestingly, GJ channels, or Cx, also interact with other membrane proteins in specialized membrane regions of the endothelial (and smooth muscle) cells. For example, Cx directly interact with caveolin and localize in the caveolae (86). In addition to Cx, many membrane receptors (e.g., endothelin receptors and 5-HT receptors) ion channels (e.g., TRPC1/C3/C6 channels, K_v1.5/K_v1.2 channels, and K_{Ca} channels), and transporters (e.g., NCX1) are also localized in caveolae in pulmonary vascular smooth muscle and endothelial cells. It is important to investigate whether and how Cx functionally interact with other membrane receptors, channels, and transporters in caveolae. Recent studies indicate that connexons, in addition to forming GJ channels, can also function as a nucleotide transporter that causes the release of ATP from cytoplasm to the extracellular space (and possibly to the caveolae space), where ATP activates purinergic receptors on adjacent cells (and on the same cells) to initiate Ca²⁺ transients (101). The connexon-mediated “paracrine” and “autocrine” effect may play an important role in the regulation of pulmonary vasoconstriction/vasodilation and vascular remodeling.

Functional Roles of K⁺ Channels and Intracellular K⁺

Ion channels form a complex signaling system functioning to maintain a cellular homeostasis: a balance between apoptosis and proliferation; between contraction and relaxation; between cell shrinkage and cell swelling and control of cellular excitability and membrane potential. Alterations in the expression and function of ion channels are a significant factor in the mechanisms leading to the development and progression of PAH.

Role of K⁺ channels in regulating PASMC contraction

As described earlier, activity of K⁺ channels, such as K_v and K_{2P} channels, is involved in regulating membrane potential in PASMC. When K_v channel closes, membrane depolarizes, whereas K_v channel opens, membrane repolarizes or hyperpolarizes. Inhibition of K⁺ channels by 4-AP in PASMC reversibly decreases whole-cell $I_{K(V)}$, causes membrane depolarization, opens VDCC, generates Ca²⁺-dependent action potentials, augments Ca²⁺ influx, increases [Ca²⁺]_{cyt}, induces PASMC contraction, and causes pulmonary vasoconstriction (Fig. 15), whereas opening of K⁺ channels (e.g., by nitric oxide) causes membrane hyperpolarization or repolarization, closes VDCC, decreases [Ca²⁺]_{cyt}, induces PASMC relaxation, and ultimately causes pulmonary vasodilation. The vasodilators that cause pulmonary vasodilation by, at least in part, opening K⁺ channels include NO, PGI₂, K⁺ (5-10 mM), EETs (49), adenosine, and cromakalim. The vasoconstrictors that have inhibitory effect on K⁺ channels include ET-1, 5-HT, high extracellular K⁺ (>15-20 mM), and 4-AP (65,235, 256-258).

Role of K⁺ channels in apoptosis in PASMC

Maintaining the proper balance between cell apoptosis and proliferation is required for normal tissue homeostasis; when this balance is disrupted, disease, such as PAH, can result. Activity of K⁺ channels plays a major role in regulating the PASMC population in the pulmonary vasculature, as they are involved in cell apoptosis, survival and proliferation.

K⁺ fluxes have been implicated in both early and late stages of apoptosis. AVD, one of the earliest morphological changes observed in cells undergoing apoptosis, is a requisite for apoptosis (37). The actual cell shrinkage or AVD is accomplished in nearly the same manner as regulatory volume decrease (RVD). As K⁺ efflux through open K⁺ channels increases in early stages of AVD, Cl⁻ ions follow, moving down their electrochemical gradient. Water exits the cell through AQP_s to maintain the osmotic pressure balance between the intracellular and extracellular compartments, thus achieving cell shrinkage (Fig. 16). Given the similarity of the molecular mechanisms of AVD and RVD, it is not surprising that in certain instances AVD is coupled to facilitated RVD (171). AVD occurs within minutes to a few hours of apoptotic induction and occurs before caspase activation, DNA fragmentation, and, in some instances, disruption of the mitochondrial membrane potential (35, 36, 38, 140, 171, 224). In some studies, K⁺ loss was observed to occur only in cells in which the mitochondrial membrane potential had already been disrupted; however, as indicated by several studies, DNA fragmentation was observed only in cells that had undergone K⁺ leakage (70).

Interestingly, some studies have found that certain apoptotic characteristics, such as cell shrinkage, K^+ efflux, and altered $\delta\Psi_m$ can be independent of both DNA degradation and caspase activity. In PASM, staurosporine (ST) augments $I_{K(V)}$, induces $\delta\Psi_m$ depolarization, and causes apoptosis (140, 229). However, when cells were exposed to a high concentration of external K^+ (which inhibits K^+ efflux by reducing the driving force for K^+), ST-induced $\delta\Psi_m$ still occurred, but the ST-induced $I_{K(V)}$ increase and apoptosis were both inhibited. Furthermore, cytochrome *c*, when introduced into PASM cytoplasm, was found to increase K^+ currents before inducing nuclear breakage; the rise in K^+ current was independent of caspase-9 activity.

The requirement of K^+ efflux for apoptosis was shown in many cell types including PASM (139, 140). In cells treated with various apoptotic inducers, including Fas ligand, dexamethasone, ST, or anisomycin, apoptosis is inhibited when K^+ efflux is prevented, either by raising the extracellular K^+ concentration (i.e., decreasing the driving force on K^+ efflux) or by pharmacologically blocking K^+ channels. Additionally, PKC has been shown to be inhibitory toward Fas ligand-induced apoptosis through its effects on K^+ loss and cell shrinkage (100).

Although increases in K^+ channel activity have been widely associated with apoptotic induction, the converse has also been observed. Apoptosis is a dynamic process, with the final biochemical changes, such as DNA degradation, marking the common manifestations of an apoptotic cell. Given the complexity of K^+ channels, it is likely that the exact mechanisms leading up to the final common stages of apoptosis are both highly cell- and inducing agent- specific. In rat PASM, overexpression of the rat $K_v1.5$ channel gene (*KCNA5*) enhances caspase activation and apoptosis (41).

The identity of the K^+ currents and channels involved in AVD has been mostly inferred from pharmacological blockade of specific K^+ channels; these studies have implicated 4-AP and TEA-sensitive K_v channels (32, 78, 139-141, 310), quinine- and Ba^{2+} -sensitive K^+ channels (171, 198), and K_{Ca} channels (78, 140, 141, 198). For example, FCCP, a mitochondrial protonophore, was shown to cause apoptosis by increasing K_{Ca} currents ($I_{K(Ca)}$) through BK_{Ca} channels in rat and human PASM; blockage of these channels with IBTX or TEA, K_{Ca} channel blockers, inhibited FCCP-induced apoptosis. Furthermore, cells lacking $K_v1.3$ are resistant to actinomycin D-induced apoptosis, failing to demonstrate DNA fragmentation, release of cytochrome *c*, and depolarization of $\delta\Psi_m$; retransfection of the missing channel restores the apoptotic response to actinomycin D. In PASM, K_{Ca} channel opening enhances nitric oxide-mediated apoptosis, and overexpressing $K_v1.5$ enhances both basal and ST-induced apoptosis (41), whereas K^+ channel block with 4-AP, TEA, or IBTX inhibits ST- and NO-induced apoptosis. These studies, taken together, indicate an essential role for increased K^+ flux, most notably through K_v and K_{Ca} channels, in apoptosis.

Inhibitory effect of intracellular K^+ on caspases

It has been shown that intracellular K^+ , in addition to contributing to the maintenance of cell volume, is inhibitory toward apoptosis in that physiological levels of K^+ inhibit the activation of caspases. Just as blockade of K^+ efflux diminishes AVD, it has also been shown to prevent the activation of cytosolic caspases. It was found that only cells with decreased

$[K^+]_{\text{cyt}}$ had intracellular caspase and nuclease activity (117). Maintaining high $[K^+]$ intracellularly suppressed the activity of these apoptotic mediators independent of the mode of apoptotic induction. Working with a cell-free system, investigators showed that in activated lysates, APAF-1 oligomerized to both an active caspase processing complex and a biologically inactive complex; in the presence of increasing $[K^+]$, the active complex did not form and caspase activation was inhibited (276). These findings were extended to show that normal intracellular levels of K^+ are enough to suppress active complex formation and that the effects of K^+ on apoptosome assembly are antagonized in a concentration-dependent manner by cytochrome *c* (47). The authors postulated that physiological $[K^+]$ levels safeguard the cell against inadvertent cytochrome *c* release which would lead to irreversible apoptosome formation. These data suggest that in addition to contributing to AVD, K^+ efflux creates a permissible environment for caspase and nuclease activity by relieving inhibition on these apoptotic mediators. Therefore, closure or block of K^+ channels, and consequent reduced K^+ efflux, would attenuate apoptosis by both preventing AVD and by maintaining caspase and nuclease inhibition (Figs. 16 and 17).

Release of cytochrome *c* from the mitochondrial inter-membrane space to the cytosol is a critical step in initiating apoptosis. It has been well documented that an increase in cytosolic cytochrome *c*, in association with Apaf-1, is a trigger for activation of caspase-9, which then activates a set of cysteine proteases (e.g., caspases-3, -5, and -7) that are central executioners of the apoptotic pathway (Fig. 17). In PASMCM, it has been reported that cytoplasmic dialysis of cytochrome *c* increases K^+ currents before inducing nuclear condensation and nuclear breakage. The cytochrome *c*-mediated increase in K^+ currents took place rapidly and was not affected by treatment with a specific inhibitor of caspase-9 (229). Cytoplasmic dialysis of recombinant (active) caspase-9 negligibly affected the K^+ currents. Furthermore, treatment of the cells with ST, an apoptosis inducer that mediates translocation of cytochrome *c* from mitochondria to the cytosol, also increased K^+ currents, caused cell shrinkage, and induced apoptosis (determined by apoptotic nuclear morphology and TdT-UTP nick end labeling assay). The ST-induced increase in K^+ currents concurred to the volume decrease but preceded the activation of apoptosis (nuclear condensation and breakage). These results suggest that the cytochrome *c*-induced activation of K^+ channels and the resultant K^+ loss play an important role in initiating the AVD when cells undergo apoptosis (Fig. 17).

Overall, K^+ fluxes contribute to apoptosis in distinct phases. The first is early AVD, in which increased K^+ efflux in response to an apoptotic mediator causes cell shrinkage. Later, a low K^+ level in the cytoplasm creates a permissible environment for caspase activation, thus leading to chromatin and protein cleavage.

Hypoxic Regulation of Ion Channel Function and Expression

K^+ channels are key regulators in cell signaling in response to hypoxia and, therefore, the development of acute HPV and chronic hypoxia-induced pulmonary hypertension. Acute hypoxia selectively inhibits K_v channels in concert with membrane depolarization and increase in $[Ca^{2+}]_{\text{cyt}}$ in canine and rat PASMCM (but not in smooth muscle cells isolated from systemic arteries) (11, 230, 320). Subsequently, investigators identified $K_v1.5$, $K_v1.2$, and

K_v2.1 as well as K_v channel β -subunits as the potential molecular correlates for the hypoxia-sensitive K_v channels in PASMC (13, 118, 215, 223). Furthermore, chronic hypoxia also decreased $I_{K(V)}$ in PASMC, while extended exposure to hypoxia (>72 hours) is known to decrease *KCNA5* gene expression in PASMC (227, 284). A distinct heterogeneity in K_v channel sensitivity to hypoxia exists which is postulated to be due to differential expression and functional sensitivity of the K_v channels to hypoxia (66, 272).

Acute-hypoxic regulation of K_v channel function

As described earlier, K_v channels are integral in the regulation of membrane potential and pulmonary vascular tone. Inhibition of K⁺ channel activity in PASMC causes membrane depolarization, which subsequently activates VDCC, leading to an increase in [Ca²⁺]_{cyt} and pulmonary vasoconstriction (Fig. 15). PASMC have a high input resistance; therefore, even a small change in K⁺ channel activity can have a significant effect on membrane potential and, consequently, on pulmonary vascular tone.

Indeed, in PASMC, acute hypoxia reversibly reduces whole-cell $I_{K(V)}$, induces membrane depolarization, and increases [Ca²⁺]_{cyt} (Figs. 18A and 18B), leading to contraction of individual PASMC (170, 195). In addition to inhibiting K_v channels, acute hypoxia also reduces K⁺ currents through K_{2P} channels and K_{Ca} channels in PASMC (129, 130, 152, 206). The acute hypoxia-mediated increase in [Ca²⁺]_{cyt} and pulmonary vasoconstriction are significantly inhibited by verapamil and nifedipine, VDCC blockers. These observations indicate that membrane depolarization-mediated activation of VDCC plays an important role in acute hypoxia-induced pulmonary vasoconstriction. Acute hypoxia may also directly increase VDCC activity independent of membrane depolarization in PASMC (92).

In addition to inhibiting K_v and K_{2P} channels, acute hypoxia can directly increase PLC, a major recourse for producing DAG that opens ROC and increases [Ca²⁺]_{cyt}. In PASMC, acute hypoxia can also activate Cl_{Ca} channels by inducing Ca²⁺ mobilization from intracellular stores (e.g., SR/ER, lysosome, and mitochondria) through (i) increases in inositol-1,4,5-trisphosphate (IP₃), cyclic ADP ribose (cADPR), and nicotinic acid adenine dinucleotide phosphate (NAADP) and (ii) inhibition of electron transfer chain in mitochondria (Fig. 18A) (48, 132, 157, 246, 292, 293, 303, 329, 331).

The precise mechanism involved in the acute hypoxia-induced inhibition of K_v channels remains unclear, but it may be related to: (i) changes in the cellular redox status or the ratio of GSG/GSSG and NADPH/NADP⁺ that affects the channel function via its regulatory β -subunit or pore-forming α -subunit; (ii) inhibition of cellular metabolism or oxidative phosphorylation with the reduced phosphate potential [ATP/(ADP+Pi)], (iii) shift of cellular metabolism from glucose oxidation, or oxidative phosphorylation to glycolysis; (iv) release or mobilization of Ca²⁺ from intracellular stores (e.g., SR/ER, lysosome, mitochondria); (v) inhibition of NADPH oxidase, (vi) inhibition of cytochrome P-450 or nonheme iron-containing moieties that are associated with or adjacent to the channel protein; (vii) indirect coupling via an intermediate locally produced by an oxygen sensor attached or adjacent to the channel protein; and/or (viii) inhibited cytochrome *c* release or increased Mg²⁺ release from mitochondria. It is currently unknown whether O₂, or changes in O₂ level, can directly

affect the movement of “gating charges” in the channel protein through the membrane electric field (315, 316).

Acute hypoxia selectively inhibits KCNA5 channels in PASM C

Acute hypoxia causes pulmonary vasoconstriction, *in vivo*, and systemic vasodilation. In isolated arterial preparation, acute hypoxia significantly increased isometric tension in pulmonary arterial rings, but had little effect on mesenteric arterial rings (321). The divergent effect of acute hypoxia on pulmonary and systemic arteries may be, at least in part, due to the selective inhibition of certain K_v channels in PASM C (but not in systemic arterial smooth muscle cells) (320). The *KCNA5*-encoded $K_v1.5$ is a pore-forming α -subunit of K_v channel family, which forms functional homo- and heterotetrameric channels. The heterotetrameric channels including one or multiple $K_v1.5$ subunit often have different electro-physiological and pharmacological properties in comparison to the $K_v1.5$ homomeric channels (118, 215, 235).

A series of experiments have been conducted to investigate (i) whether acute hypoxia inhibits homomeric *KCNA5* channels and (ii) whether the effect of acute hypoxia on *KCNA5* currents (I_{KCNA5}) is selective to PASM C. Despite a comparable amplitude and kinetic profile of I_{KCNA5} in all of the exogenously transfected cells, acute hypoxia selectively decreases I_{KCNA5} in PASM C transfected with *KCNA5* gene. Reduction of the PO_2 from 154 ± 5 to 32 ± 4 mmHg significantly inhibits the current, an effect fully reversible upon restoration of extracellular PO_2 to 145 to 150 mmHg; however, acute hypoxia has little effect on I_{KCNA5} in HEK-293, COS or MASM C (Fig. 18C). Interestingly, overexpression of *KCNA5* cause membrane depolarization, while 4-AP decreases I_{KCNA5} , in all cell types (PASM C, MASM C, HEK-293, and COS cells) transfected with *KCNA5*. These observations imply that (i) *KCNA5* is an important K_v channel α -subunits that contributes to regulating resting membrane potential, and (ii) the selective inhibitory effect of acute hypoxia on *KCNA5* channel in PASM C (relative to systemic arterial smooth muscle cells) may play an important role in hypoxia-induced membrane depolarization and pulmonary vasoconstriction (41, 223).

Chronic hypoxic regulation of Ion channel expression

While acute hypoxia decreases whole-cell I_K in PASM C by inhibiting K^+ channel activity, chronic exposure of PASM C to hypoxia may affect the transcription and translation of K^+ channel genes and cause posttranslational modifications, trafficking, and degradation of the channel proteins. The chronic hypoxia-mediated transcriptional and translational regulation of K^+ channels may result from the direct effect via hypoxia-sensitive transcription factors (e.g., HIF-1 α and -2 α) and/or the indirect through molecular and cellular changes (e.g., redox changes and metabolic alterations) induced by acute hypoxia.

Downregulation of K^+ channels in chronic hypoxic pulmonary hypertension—

In a chronic hypoxic environment, not only the activities of K^+ channels are inhibited, but most importantly the expressions of K_v channels are also downregulated (Fig. 19). Actually, the resting E_m of PASM C from chronically hypoxic animals is significantly less negative (i.e., membrane is depolarized) in comparison to that in normoxic animals, which is

accompanied by significantly reduced $I_{K(V)}$. Using an *in vitro* model, Wang et al. demonstrated that prolonged exposure of rat PASMC to hypoxia significantly downregulated mRNA and protein expression level of two specific K_v channel α -subunits ($K_v1.2$ and $K_v1.5$) (284). In addition, decreased mRNA expression of $K_v1.1$, $K_v2.1$, $K_v4.3$, and $K_v9.3$ α -subunits in cultured rat PASMC under chronic hypoxia was reported and confirmed by several research groups (13, 105, 177, 209, 227, 286). The response of downregulation of K_v channels is specific to PASMC and therefore selective for the pulmonary circulation since, until now, chronic hypoxic inhibition of the expression of K_v channels α - or β -subunits have not been reported in MASM. Two animal models including the chronically hypoxic animal model and the $K_v1.5$ knockout mouse model have been set up and studies based on these two models have emphasized the importance of K_v channels in the pulmonary vascular response. Investigations of freshly isolated PASMC from chronically hypoxic animal models show downregulation of $K_v1.2$ and $K_v1.5$ and a decrease in the oxygen-sensitive component of I_K (254, 257, 286). Studies with $K_v1.5$ knockout mice models show impaired HPV and reduced oxygen-sensitive K^+ current in PASMC. All these research provide strong evidence for the role of K_v channels in the chronic pulmonary vascular response to hypoxia.

The mechanisms by which the expression of K_v channels is downregulated have not been totally understood yet. Many hypotheses have been put forward to explain the chronic hypoxia-induced inhibition of K_v channels expression in PASMC, which include the following: (i) upregulation or downregulation of the transcription factors and signal transduction proteins that can directly bind to the K_v channel gene promoters (e.g., KRE) and modulate the K_v channel gene transcription (234, 255, 297); (ii) induction of transcription factors that upregulate intermediate inhibitors of the K_v channel genes, such as ET-1 (297). Phosphorylation and/or redox modification of responsive transcription factors are the most common pathways that are involved in the transduction of hypoxic signals from the sensor to appropriate gene expression. A variety of transcription factors and signal transduction signaling proteins such as HIF-1, nuclear factor- κ B, c-fos/c-jun, BMP (bone morphogenetic protein), P53, KBF, FixL, and FixJ can be modulated by hypoxia, suggesting that a large number of transcriptional pathways contribute to the response under chronic hypoxic environment. For example, it has recently been reported that overexpression of *c-jun* gene can significantly decrease whole-cell $I_{K(V)}$, downregulates the mRNA expression of the $K_v1.5$ channel subunit, and accelerates $I_{K(V)}$ inactivation in cultured PASMC (313). There is also a large amount of data indicating that the *c-jun* family of genes is inducible by hypoxia (7), suggesting that the *c-jun* may indirectly downregulate transcription of $K_v1.5$ gene and decrease $I_{K(V)}$.

In addition to inhibiting K_v channels, chronic hypoxia also affects other types of channels, including downregulation of K_{2P} channels and upregulation of Cl^- channels (e.g., bestrophin). The membrane depolarization due to down-regulated K^+ channels and upregulated Cl^- channels, open VDCC, promotes Ca^{2+} influx, increases $[Ca^{2+}]_{cyt}$, and stimulates PASMC contraction and proliferation. Furthermore, chronic hypoxia also upregulates VDCC (specifically α_{1C} subunits) (112), there are more VDCC available to be open when cells are depolarized (Fig. 19).

Upregulation of TRPC in chronic hypoxic pulmonary hypertension—

Development of chronic hypoxic pulmonary hypertension is associated with elevated resting $[Ca^{2+}]_{cyt}$ in PASMC and contraction of pulmonary vascular smooth muscle. It has been widely accepted that the maintenance of increased PASMC $[Ca^{2+}]_{cyt}$ and tone during chronic hypoxia requires Ca^{2+} influx through pathways other than VDCC. As it has been discussed earlier that SOC which are believed to be composed of TRPC proteins may play very important roles in response to acute hypoxia, it is likely that SOC also contribute to the development of chronic hypoxic pulmonary hypertension. Evidence provided by several research groups has confirmed that exposure to chronic hypoxia caused enhanced activity of SOC (156, 165, 236, 285), which was required for maintenance of elevated resting $[Ca^{2+}]_{cyt}$ and active tone and correlated with elevated expression of TRPC proteins (especially TRPC1/6) and Orai/STIM (Fig. 19) (164).

Compared with K_v 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 chronic hypoxia. The research data showed that the development of right ventricular hypertrophy, and vascular remodeling was delayed in *Hif1a*^{+/-} compared with *Hif1a*^{+/+} mice (308). Similar studies now provide compelling evidence that the developing stories of HIF-1 and TRPC in the pulmonary vascular response to chronic hypoxia form part of the same narrative (285, 287). The authors first confirm previous observations of elevated $[Ca^{2+}]_i$ and CCE (or SOCE) in transiently cultured PASMC from rats made hypoxic for 21 days. They then show that *in vivo* chronic hypoxia in both rats and control mice results in an upregulation of the expression of protein and mRNA for TRPC1 and TRPC6, but not TRPC4. Crucially, they demonstrate that the increases in basal $[Ca^{2+}]_i$ and TRPC1/6 expression are absent in *HIF1a*^{+/-} mice. Accordingly, overexpression of a constitutively active form of HIF-1 α also increases the expression of TRPC1/6, but not TRPC4 (285).

As described in this section, acute hypoxia induces pulmonary vasoconstriction and chronic hypoxia causes structural changes and remodeling in pulmonary arteries, eventually leading to the chronic pulmonary hypertension and right ventricular hypertrophy. In both cases, the increases in $[Ca^{2+}]_{cyt}$ 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 K_v channels, VDCC and SOC, will have been changed, either “turned on” or “turned off.” The functional changes of these ion channels are not totally separated or individual events and may have effects on one another. The effects of acute hypoxia on both the ion channels and the intracellular Ca^{2+} concentration are reversible once the “alarm” of the absence of oxygen has been spared and the cells are treated under regular oxygen concentration. However, chronic hypoxia has long-time and irreversible effects upon ion channels and $[Ca^{2+}]_{cyt}$ due to sustained absence of oxygen. Under chronic hypoxic conditions, both mRNA and protein expression levels of the ion channels involved have been altered, providing the basis for the sustained effects. Decreased mRNA expression of $K_v1.1$, $K_v1.2$, $K_v1.5$, $K_v2.1$, $K_v4.3$, and $K_v9.3$ α -subunits in cultured rat PASMC under chronic hypoxia has been confirmed. However, the mechanisms by which the expressions of K_v channels are downregulated are not completely understood. It has been proposed that a

variety of transcription factors and signal transduction proteins that can directly bind to the K_V channel gene and modulate the K_V channel gene transcription; induction of transcription factors that upregulate intermediate inhibitors of the K_V channel genes such as ET-1. Compared with K_V channels, 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 using the rat model. Interestingly, a study using the mouse model shows that TRPC6 is essential for acute hypoxia-induced pulmonary vasoconstriction and gas exchange, but may not be involved in chronic hypoxia-induced pulmonary hypertension because both wild-type and TRPC6 knockout mice develop pulmonary hypertension during chronic hypoxia (74).

Microdomains and Organized Signaling in Regulating Ion Channel Function

Caveolin and caveolae

Caveolae are 50 to 100 nm flask-shaped invaginations of the plasma membrane. Biochemically, caveolae represent a subdomain of the plasma membrane enriched in cholesterol, sphingolipids, and a family of integral membrane proteins named caveolins. Caveolins are a family of 21 to 24 kDa integral membrane proteins with three mammalian isoforms identified as Cav-1, caveolin-2 (Cav-2), and caveolin-3 (Cav-3) (50, 79-81). Cav-1 is most prominently expressed in endothelial, fibrous, and adipose tissue. The expression pattern of Cav-2 is similar to that of Cav-1, which indicates Cav-2 may be co-expressed with Cav-1. The expression of Cav-3 is restricted to striated and smooth muscle. The caveolae formation was greatly suppressed in the vascular smooth muscle cells in Cav-1 knock-out mice (154), while this formation remained present in striated muscle types suggesting that Cav-1 is required for caveolae formation in smooth muscle and that Cav-3 cannot compensate for the physiological function of Cav-1 in smooth muscle cells. Additionally, in Cav-3 knockout mice, skeletal and heart muscle lack caveolae, whereas smooth muscle still demonstrated formation of caveolae in-vaginations. Therefore, it is reasonable to predict that Cav-1 is the major structural caveolin isoform in smooth muscle and that Cav-2 and Cav-3 may play roles other than simply formation of caveolae, such as modulation of Cav-1 expression, modulation of cell signaling and modulation of metabolism.

Colocalization of caveolins and ion channels in caveolae

Investigations about the ion channels and related signaling pathways localized in caveolae in PASM and endothelial cells will be helpful in determining their roles in the pulmonary circulation. It is estimated that the caveolae, by virtue of enrichment in signaling molecules and ion channels, would be key in excitation-contraction coupling and perhaps in O_2 -sensing or susceptibility to chronic hypoxia-induced pulmonary hypertension (193).

The recruitment of signaling molecules to caveolae can determine Ca^{2+} sensitization in smooth muscle in response to K^+ depolarization and receptor-agonists (194, 233). It has been documented that the K_V channel, $K_{V1.5}$, targets to caveolae and co-localizes with caveolin (178, 179). The latter studies demonstrated that a cholesterol-binding agent 2-hydroxy-propyl- β -cyclodextrin (β -CD) which disrupts caveolae, induced a hyperpolarizing

shift in the voltage dependence of activation and inactivation. The large conductance K_{Ca} channels are also associated with Cav-1 in vascular endothelial cells indicated by immunoprecipitation and glutathione *s*-transferase pull-down assays (289). These and other data suggest that Cav-1 is a regulator of K^+ channel function.

Ca^{2+} influx across the plasma membrane following depletion of the intracellular Ca^{2+} stores is known as SOCE. As described above, a relation between SOCE and the members of the TRPC family of proteins has recently emerged. TRPC1, TRPC3 and TRPC4 have been suggested to associate with caveolae via interaction with Cav-1. Bergdahl et al. have demonstrated that TRPC1 is functionally coupled to ET-1-type receptors in intact arteries and that it is localized to caveolae (24, 144). Additionally, disruption of caveolae and lipid rafts by cholesterol depletion resulted in increased sensitivity of TRPC1 to SOCE and ET-1 responses. A recent report by the same group demonstrated that the plasticity of TRPC expression is enhanced by vascular injury in arterial smooth muscle, that TRPC expression correlates with cellular Ca^{2+} handling, and that TRPC1 is a subunit that is highly up-regulated by SOCE channels (24).

Pathogenic Role of Ion Channels in the Development and Progression of Pulmonary Arterial Hypertension

In addition to the synthetic, structural, and functional abnormalities in the pulmonary vasculature, substantial and convincing evidence has recently emerged that point to multiple derangements in complex membrane receptors, ion channels, and intracellular signaling pathways that can contribute to the manifestation of pulmonary vascular disease, especially in IPAH. It is now generally accepted that this disease involves a heterogeneous constellation of multiple genetic, molecular and humoral abnormalities that all share a common end result, that is, pulmonary vascular remodeling. Dysfunction of K_v channels and abnormality of intracellular Ca^{2+} homeostasis are examples of cellular factors involved in excessive pulmonary vascular remodeling in patients with IPAH.

Pathophysiological and pathological features of the pulmonary vasculature in patients with pulmonary arterial hypertension

As discussed earlier, sustained pulmonary vasoconstriction, excessive pulmonary vascular remodeling and *in situ* thrombosis are the major causes for the elevated PVR in patients with IPAH. Pulmonary vascular wall is structurally characterized of three layers: adventitia (mainly consists of fibroblasts and extracellular matrix), media (or smooth muscle), and intima (or the endothelium). Pulmonary vascular remodeling is characterized by adventitial and medial hypertrophy due to fibroblast and smooth muscle cell growth and hyperplasia, intimal and plexiform lesions that obliterate the intralumen in small arteries, muscularization of precapillary arterioles, and *in situ* thrombosis that causes intraluminal fibrosis and obliteration (Fig. 20A). Decreased compliance (or increased stiffness) of pulmonary vascular wall, due to fibroblast proliferation and extracellular matrix abnormalities, is the major cause for the decreased distention and recruitment of pulmonary vessels observed in patients with pulmonary hypertension (Fig. 20A). An increased afterload for right heart, due to elevated

resistance to the pulmonary circulation, in patients with IPAH has long been known as a cause of right ventricular hypertrophy, right heart failure and death in patients with IPAH.

The pulmonary arteriogram or angiogram clearly shows pruning of the lobular vessels (82), which is mainly due to narrowing (sustained vasoconstriction) or occlusion (vascular remodeling) of small arteries and muscularization of precapillary arterioles (Fig. 20B, upper panels). By histological examination in normal subjects, three kinds of pulmonary arteries are recognized: elastic arteries, muscular arteries, and arterioles. In patients with IPAH, intimal thickening in the elastic arteries is more noticeable than in normal subjects, while medial hypertrophy is often found in muscular arteries and muscularization in arterioles. Thrombosis of segmental arteries is a common histological or pathological finding in patients with IPAH, which is often involved in muscular arteries. The most significant histological changes in IPAH patients are in the muscular arteries and arterioles. These vessels are often partially or completely occluded (or obliterated) by an abnormal sparsely cellular tissue that consists in fibroblasts, myofibroblasts, smooth muscle cells, and endothelial cells (Fig. 20B, lower panels).

Similar to the systemic vascular remodeling (Fig. 21A), pulmonary vascular structural changes can also be divided to the following categories: (i) obliteration of small arteries due to intraluminal fibrosis, intimal, and plexiform lesions, (ii) *in situ* thrombosis in muscular small arteries, (iii) concentric hypertrophy (or medial thickening) due to smooth muscle proliferation, (iv) inward entrophy due to sustained vasoconstriction, and (v) eccentric hypertrophy and stiffen due to fibroblast proliferation and collagen deposition in adventitia and extracellular matrix (Fig. 21B). Therefore, pruning of the lobular vessels showing in pulmonary angiogram is caused by multiple mechanisms: sustained vasoconstriction, concentric remodeling, intraluminal fibrosis and obliteration, muscularization of precapillary arterioles, *in situ* thrombosis, and lost of capillaries. Downregulation of K_v channels, upregulation of TRPC, and abnormality of intracellular Ca^{2+} homeostasis are examples of cellular factors involved in sustained pulmonary vasoconstriction and excessive pulmonary vascular remodeling in IPAH patients.

Dysfunctional K^+ channels and pulmonary arterial hypertension

Defective (and downregulated) K^+ channels have been implicated in pulmonary hypertension (87, 317, 323). In PASMC from patients with IPAH, $I_{K(V)}$ is significantly attenuated in comparison to that in control cells. The anorexic agent fenfluramine can cause pulmonary hypertension. In PASMC, fenfluramine reduces the 4-AP-sensitive K^+ current, depolarizes E_m , and increases $[Ca^{2+}]_{cyt}$. These observations fit the view that dysfunctional K^+ channels in PASMC may play an etiologic role in the development of PAH.

Decreased K_v channel activity leads to pulmonary vasoconstriction and PASMC proliferation—As described earlier, in native cells, functional K_v channels are heteromultimeric tetramers composed of two subunits, the pore forming α -subunit and the regulatory β -subunit. In PASMC from IPAH patients, the amplitude of whole-cell $I_{K(V)}$ and mRNA/protein expression level of K_v channel α -subunits (e.g., $K_v1.2$ and $K_v1.5$) are both significantly decreased in comparison to PASMC from patients with non-pulmonary

hypertension (NPH) cardiopulmonary diseases (317, 323). The downregulated K_v channels and decreased $I_{K(V)}$ associate with a more depolarized E_m in IPAH-PASMC. In smooth muscle cells, the voltage window for activation of plasmalemmal L-type VDCC and sustained elevation of $[Ca^{2+}]_{cyt}$ ranges between -40 and -15 mV. Therefore, the membrane depolarization that occurs in PASMC from IPAH patients would cause a sustained increase in $[Ca^{2+}]_{cyt}$ by promoting CCE through opened VDCC. Indeed, in PASMC from IPAH patients, the resting $[Ca^{2+}]_{cyt}$ is much higher than in PASMC from normotensive control patients. In summary, downregulated K_v channel expression and decreased whole-cell $I_{K(V)}$ in PASMC from IPAH patients may be important for elevating $[Ca^{2+}]_{cyt}$ by activating VDCC. The increased $[Ca^{2+}]_{cyt}$ in PASMC, as mentioned earlier, not only causes pulmonary vasoconstriction, but also stimulates PASMC proliferation, and ultimately contributing to the development of pulmonary vascular remodeling (Fig. 22).

Decreased K_v channel activity leads to inhibition of AVD and apoptosis—As discussed earlier, AVD is an early hallmark of cell undergoing apoptosis. Loss of cytosolic K^+ ($[K^+]_{cyt}$) is in part responsible for AVD. Activation of K^+ channels leads to efflux or loss of K^+ which induces or accelerates AVD and apoptosis. Conversely, inhibition or decreased activity of K^+ channels has been shown to cause accumulation of K^+ in the cells, maintaining $[K^+]_{cyt}$ sufficiently high to decelerate AVD and inhibit apoptosis. Furthermore, maintenance of sufficient $[K^+]_{cyt}$ appears to be also required for suppressing the activity of caspases and nucleases. In short, diminished $[K^+]_{cyt}$ due to increased K^+ efflux through plasmalemmal K^+ channels results in cell shrinkage and activation of cytoplasmic caspases; while maintenance of sufficient K^+ in the cytosol due to decreased activity of K^+ channels inhibits apoptosis. In PASMC from IPAH patients, apoptosis induced by BMP and ST is inhibited in comparison to cells isolated from control patients (325). Furthermore, overexpression of $K_v1.5$ (*KCNA5*) gene in PASMC increases whole-cell $I_{K(V)}$, accelerates ST-mediated apoptotic cell shrinkage and enhances apoptosis (41). These data suggest that inhibited apoptosis in IPAH-PASMC may be related to the decreased K_v channel activity. The downregulated K_v channels in PASMC from IPAH patients may be a shared mechanism involved in mediating pulmonary vasoconstriction and stimulates PASMC proliferating by increasing $[Ca^{2+}]_{cyt}$, and in inhibiting apoptosis by decreasing K^+ loss or maintaining cytoplasmic $[K^+]$ (Fig. 22).

Impaired mitochondrial fusion and inhibited mitofusin proteins have been recently identified in PASMC from different forms of pulmonary hypertension. Impaired mitochondrial fusion, together with pathological activation of pyruvate dehydrogenase kinase and deficiency in superoxide dismutase-2 (SOD2), inhibits the proximal electron transport chain and decreases mitochondrial ROS production (e.g., H_2O_2), and leads to downstream pathway that promotes proliferation and impairs apoptosis in PASMC by activating HIF-1 α and downregulating K_v channel expression (e.g., *KCNA5*). The mitochondrial-ROS-HIF1 α -*KCNA5* pathway may well be an important mechanism involved in the inhibited expression and function of K_v channels in PASMC from patients with PAH (10, 33, 34).

Dysfunctional BMP signaling is associated with decreased K_v channel activity and inhibited PASMC apoptosis—Mutations in the BMP-R2 gene (located at

chromosome 2q33) have been associated with inheritable PAH as well as in 15% to 25% of patients with IPAH (149, 167). BMPs are signaling molecules that belong to the transforming growth factor- β (TGF- β) superfamily and play an important role in regulating cell proliferation, differentiation, and apoptosis (180, 181, 189, 192). Binding of BMP ligand (e.g., BMP-2 and -7) to either of the receptors leads to hetero-oligomerization of BMP-R1 and BMP-R2 and formation of ligand-receptor complex, which in turn activates the downstream signaling elements such as the receptor-activated Smad proteins. The activated BMP-R1 phosphorylates the R-Smad proteins (e.g., Smad-1, -5, and -8), which is then dimerized with co-Smad (e.g., Smad-4) forming a complex signaling protein that can translocate into the nucleus. Furthermore, activation of Smad proteins is inhibited by overexpression of calmodulin, a Ca^{2+} -sensitive cytosolic protein. This attenuates the response of TGF- β /BMP signaling transduction suggesting that elevated $[\text{Ca}^{2+}]_{\text{cyt}}$ can lead to activation of calmodulin, which inhibits Smad proteins, having an inhibitory effect on TGF- β /BMP signaling pathway and TGF- β /BMP-mediated apoptosis in human PASMC (Fig. 23A).

A number of factors can modulate PASMC proliferation and apoptosis, including transcription factors, growth factors, and mitogens. BMP-2-mediated apoptosis was associated with transient phosphorylation of Smad1, a protein signaling element activated upon binding of BMPs to their type I and II (BMP-RI and -RII) receptors, and marked downregulation of Bcl-2, an antiapoptotic protein. In an earlier study, Bcl-2 was shown to enhance PASMC survival by, at least in part, decreasing K_v channel activity and downregulating K_v channel α -subunit expression in PASMC enhanced cell survival. This supports the mounting evidence suggesting that enhanced K^+ activity is essential to the onset and progression of PASMC apoptosis in the presence of BMP receptor ligands.

A recent study has characterized the effect of BMP-2 on $I_{\text{K(V)}}$ and K_v channel α - and β -subunit expression in human PASMC (84). Treatment of PASMC with BMP-2 (200 nM for 24 h) significantly increased $I_{\text{K(V)}}$ (Fig. 23B). BMP-2 enhanced $I_{\text{K(V)}}$ at all test membrane potentials, and current amplitude and density were ($P < 0.01$) by BMP-2 at a pulse potential of -40 mV, suggesting that BMP-2-sensitive $I_{\text{K(V)}}$ is active at potentials close to resting membrane potential in human PASMC.

Amplitude of whole-cell $I_{\text{K(V)}}$ is predominantly determined by changes in the channel function and expression. Increases in the K_v channel gene expression would thus increase the number of K_v channels and subsequently increase whole-cell $I_{\text{K(V)}}$. The real-time PCR experiments show that KCNA1 to 2, KCNA4, KCNB1 to 2, and KCNC1 expression levels were slightly decreased by BMP-2 treatment in human PASMC, as were those of KCNB1 and KCNB3 regulatory subunits. Conversely, KCNA5, KCNA10, KCNC3 to 4, KCND1 to 3, KCNF1, KCNG1, KCNS1, and KCNG3 α -subunits and KCNB2 β -subunits were slightly upregulated in the same experiment. More significant upregulation was observed for KCNA6-7 and KCNS3. The most dramatic changes observed were the downregulation of KCNG2 and KCNV2 mRNA with BMP-2 treatment. Both of these subunits encode for electrically silent " K_v channel γ -subunits" which may, upon co-expression with functional pore-forming K_v channel α -subunits, alter the electrophysiological or biophysical properties of the functional tetrameric channel.

In addition to K_v channels, BMP2 treatment also significantly increases activity of the large-conductance Ca^{2+} -activated K^+ channels (BK_{Ca}) in PASMC (Fig. 23C). These observations indicate that BMP signaling targets on multiple K^+ channel genes to enhance whole-cell I_K , which subsequently results in accelerated AVD, relieved inhibition of K^+ on intracellular caspases, and increased apoptosis in PASMC.

Pathogenic role of TRPC in pulmonary arterial hypertension

As discussed earlier, in addition to Ca^{2+} influx through VDCC, $[Ca^{2+}]_{cyt}$ can also be increased by promoting Ca^{2+} influx through ROC and SOC (Fig. 24). Therefore, the elevated $[Ca^{2+}]_{cyt}$ in IPAH-PASMC may be caused by multiple mechanisms, that is, enhanced CCE through VDCC opened by membrane depolarization due to inhibited K_v channels is only one of the important pathways for elevated $[Ca^{2+}]_{cyt}$ and its resultant sequelae in the development of pulmonary vasoconstriction and vascular remodeling in IPAH. Activation of SOC by depletion of intracellular Ca^{2+} stores has been implicated in the elevation of $[Ca^{2+}]_{cyt}$ in PASMC from IPAH patients.

TRPC are upregulated and SOCE is enhanced in PASMC from patients with pulmonary arterial hypertension—In comparison to growth-arrested cells, proliferating human PASMC exhibit a high level of resting $[Ca^{2+}]_{cyt}$ potentially maintained by a constant Ca^{2+} influx. Interestingly, normal PASMC proliferation is associated with a significant increase in mRNA and protein expression of TRPC such as TRPC1 and TRPC6 (99, 271, 312, 314). Consistent with the upregulated TRPC expression, the amplitude of SOCE is also significantly greater in proliferating PASMC than in growth-arrested cells. Inhibition of TRPC expression with antisense oligonucleotides markedly decreases the amplitude of SOCE and significantly inhibits PASMC proliferation. Levels of various growth factors, such as PDGF, are noted to be higher in lung tissues of patients with severe pulmonary hypertension. Therefore, upregulation of TRPC appear to be a critical mechanism by which growth factors mediate PASMC proliferation.

As well as its essential role in normal PA contraction and PASMC proliferation, increased SOCE, secondary to increased formation of SOC by upregulated TRPC genes, is also important in the development of pulmonary vascular remodeling in patients with IPAH (Fig. 25). Using PASMC isolated from normotensive control patients and IPAH patients undergoing lung transplant, investigators found that PASMC from IPAH patients demonstrate significantly higher resting $[Ca^{2+}]_{cyt}$ when compared to cells from control patients. In addition, the magnitude of SOCE, evoked by passive store depletion with CPA, is significantly greater in PASMC from IPAH patients than in cells from control patients (311, 312). IPAH is a heterogeneous disorder with abnormal PASMC proliferation as its central theme. These data suggest that SOCE or CCE is essential in maintaining the adequate cytoplasmic, nuclear, and SR/ER Ca^{2+} required for PASMC proliferation. Enhanced SOCE, possibly via upregulation of TRPC such as TRPC1, TRPC3, and TRPC6 may represent another critical downstream pathogenic event or mechanism involved in the development of severe pulmonary hypertension. Accordingly, interruption of SOCE at any point, from agents that downregulate TRPC gene expression to specific blockers for SOC in

PASMC, may prove beneficial in the development of therapeutic approaches for treatment of severe pulmonary hypertension.

Conclusion

Ion channels are fundamental to the maintenance of cellular homeostasis and to the excitability of cells in response to a variety of stimuli. Alterations in the expression and function of ion channels are key features in the development and pathogenesis of pulmonary vascular disease and thus ion channels and consequential signaling cascades are potential sites for therapeutic intervention.

PAH is a disease entity that defines a single predominating pathophysiological cascade theory, but rather it involves a heterogeneous constellation of multiple genetic, molecular, and humoral abnormalities. Although each abnormality is likely important in itself, none appears to be sufficient to cause the disease by itself. Interestingly, some of the derangements such as the BMP-R2 gene mutations occur in all vascular smooth muscle cells, yet it manifests as disease only in the pulmonary vascular bed. This supports the notion of multiple-hit theory in which some of the hits confer pulmonary specificity. For instance inheritance of BMP-RII gene mutation, followed by acquiring mutations in K_v channels and gain-in-function mutations in TRPC can trigger the development of severe IPAH.

Acknowledgments

This work was supported in part by grants from the National Institutes of Health (DK083506, HL066012 and HL098053). The authors would like to thank Jian Wang, Elyssa D. Burg, Carmelle V. Remillard, Oleksandr Platoshyn, Mehran Mandegar, Eun A. Ko, Michael Y. Song, Ying Yu, Ivana Fantozzi, Elena E. Brevnova, Daryoush Ekhterae, Stefanie Krick, Shen Zhang, Michele A. Sweeney, Minlin Xu, Judd W. Landsberg, Joseph R.H. Mauban, Aiko Ogawa, Sharon S. McDaniel, and Beth R. Lapp for their contributions to this work

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Further Reading

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Figure 1. Pulmonary angiogram (left) and cast (right) of a segment of arterial tree in human lung (A), and the proposed three schemes describing this complex structure by the Weibel model (B), the Strahler model (C), and the diameter-defined Strahler's system (D).

Figure 2.

Electric circuit of model cell membrane and intracellular ion homeostasis. Single-section (A) and parallel-conductance (B) model of cell membrane. For the parallel-conductance model, one assumes independent conductance channels for K^+ , Na^+ , and Cl^- . (C) Schematic diagram showing major ion channels and transporters that determine membrane potential as well as electrical and chemical gradients for K^+ , Na^+ , Ca^{2+} , and Cl^- in pulmonary artery smooth muscle cells (PASMC). Resting membrane potential (E_m) for a PASMC is -40 to -60 mV. E_K , E_{Na} , E_{Ca} , and E_{Cl} represent the equilibrium potentials for K^+ , Na^+ , Ca^{2+} , and Cl^- , respectively. The size of the arrows indicates the driving force for the given ions. Inset, a diagram showing the channel protein embedded in the lipid membrane.

Figure 3. Regulation of intracellular $[Ca^{2+}]$ (A) and the major causes of membrane depolarization (B) in pulmonary artery smooth muscle cells. SERCA, Ca^{2+} - Mg^{2+} ATPase (Ca^{2+} pump) in the SR/ER membrane; PM, Ca^{2+} - Mg^{2+} ATPase (Ca^{2+} pump) in the plasma membrane; NCX, Na^{+} - Ca^{2+} exchanger; RyR, ryanodine receptor; IP₃R, inositol 1,4,5-trisphosphate (IP₃) receptor; E_m , membrane potential.

Figure 4.

Topological structure of the K_v channel α -subunit (A) and voltage-dependent Ca^{2+} channels (VDCC) (B). (A) A planar representation of a K_v channel α -subunit (a) and its interaction with other α -subunits (b) and the β -subunits (c) via the N-terminal T1 domain. The graphs in b and c show a 4α -tetramer and an $4\alpha/4\beta$ -octamers, respectively. The pore of the 4α -tetramer is shown in b. (B) A planar representation of a VDCC subunit. Inset: diagrammatical representation of a VDCC channel in the plasma membrane with associated subunits. The pore region (P) forming between the S5 and S6 subunits is indicated in red.

Figure 5.

A rise in $[Ca^{2+}]_{cyt}$ is a major trigger for pulmonary vasoconstriction. When $[Ca^{2+}]_{cyt}$ rises in pulmonary artery smooth muscle cells, Ca^{2+} binds to calmodulin (CaM), which causes contraction by activating (or phosphorylating) myosin light chain kinase (MLCK) and indirectly causes the inhibition of myosin light chain phosphatase (MLCP) via Rho kinase (ROK). Activation of G protein-coupled receptors (GPCR) can also cause Ca^{2+} -independent contraction (or Ca^{2+} sensitization) through the RhoA/ROK pathway.

Figure 6.

A rise in $[\text{Ca}^{2+}]_{\text{cyt}}$ is an important stimulus of pulmonary artery smooth muscle cells proliferation. In addition to activating MLCK and causing contraction, cytosolic Ca^{2+} also activates CaM kinase (CaMK) and mitogen-activated protein kinase (MAPK), as well as various transcription factors (e.g., NFAT, CREB, AP-1 family members, and NF- κ B), to facilitate cell passage through the cell cycle and cause cell proliferation. The arrows in the inset indicate the Ca/CaM-sensitive steps in the cell cycle. MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; PVR, pulmonary vascular resistance; PAP, pulmonary arterial pressure; SR/ER, sarcoplasmic (or endoplasmic) reticulum.

Figure 7. Structure of transient receptor potential channel (TRP). (A) Planar views of the TRPC1, TRPV1, and TRPM1 channel subunits. (B) Representative traces reflecting changes in $[Ca^{2+}]_{cyt}$ (left panel) recorded in pulmonary artery smooth muscle cells (PASMC) before and during application of 10 μM cyclopiazonic acid (CPA), a SERCA inhibitor that depletes intracellular Ca^{2+} stores. 0 Ca^{2+} , Ca^{2+} -free solution. The rise in $[Ca^{2+}]_{cyt}$ due to store-operated Ca^{2+} entry (SOCE) or capacitative Ca^{2+} entry (CCE) is indicated in the shadow area. The right panel shows representative whole-cell currents recorded in human PASMC cells before (-CPA) and after (+CPA) depletion of intracellularly stored Ca^{2+} from the SR using 10 μM CPA.

Figure 8.

Oligomerization and translocation of STIM to SR/ER-plasma membrane junctions are the major mechanism involved in SOCE through Orai tetramer-formed SOC. (A) The Ca²⁺ sensor EF-hand domain in the N terminus of STIM has bound Ca²⁺ (red circles) when the SR/ER Ca²⁺ store is filled with Ca²⁺ (up to 1 mM). Depletion of Ca²⁺ from the SR/ER causes Ca²⁺ to unbind from the low-affinity EF-hand of STIM, which subsequently leads to STIM oligomerization and translocation to the SR/ER-plasma membrane junctions. (B) STIM accumulation in the vicinity of Orai channel dimers induces Orai channels to cluster in the adjacent plasma membrane. The C-terminal effector domain of STIM causes Orai channels to open by direct binding of the distal coiled-coil domain, and triggers Ca²⁺ entry. Two STIMs can activate a single SOC formed by an Orai tetramer; channel activation of Orai may involve a preliminary step of assembling Orai dimers into a functional tetramer.

Figure 9.

Functional coupling of receptors, transient receptor potential channel (TRPC) and NCX in caveolae in pulmonary artery smooth muscle cells (PASM). (A) Electron microscopy graphs showing the structure of plasma membrane in PASM isolated from normal subjects and patients with idiopathic pulmonary arterial hypertension (IPAH). IPAH PASM have increased flask-like invaginations of the plasma membrane consistent with the morphology of caveolae (indicated by arrows) and as determined by the number of caveoli per membrane length. (B) and (C) Schematic diagram depicting the caveolin-1 binding domains of TRPC1 (B) and the potential mechanisms (C) that are involved in ligand-mediated regulation of intracellular $[Ca^{2+}]_{\text{cyt}}$ through G protein-coupled receptors (GPCR), TRPC, and Na^+/Ca^{2+} exchangers (NCX) in caveolae. CBM, cav1-binding motif; PBD, protein 4-binding domain; CSD, caveolin-scaffolding domain; PM, the plasma membrane; ER/SR, endoplasmic reticulum and sarcoplasmic reticulum; G, G protein; IP₃, inositol 1,4,5-trisphosphate; IP₃R, IP₃ receptor; SERCA, SR/ER Ca^{2+} - Mg^{2+} ATPase; Cav-1, caveolin-1.

Figure 10.

Structure of K^+ channels. Planar membrane topologies of single K^+ channel subunits for voltage-gated K^+ (K_v) channels (A), Ca^{2+} -activated (BK_{Ca}) K^+ channels (B), inward rectifier K^+ (K_{IR}) channels (C), and two-pore domain (K_{2p}) K^+ channels (D), respectively. The pore-forming loop is indicated and the voltage sensor in the transmembrane (TM) domain 4 for K_v and BK_{Ca} channels. Membrane topology of a K_{2p} channel subunit featuring two pore regions, P1 and P2 and four TM-spanning domains, M1 to 4 and cytoplasmic N- and C- termini (D). The ATP-sensitive K^+ (K_{ATP}) channels are heterooctamers formed by the pore-forming K_{IR} subunits and the regulatory subunits, SUR (e.g., Kir6.1/SUR2B and Kir6.2/SUR2B) (as shown in C).

Figure 11.

Structure of voltage-gated Na⁺ channels. (A) Planar schematic diagram showing structural arrangement of Na⁺ channel α and β₁/β₂ subunits. (B) Representative currents showing inward Na⁺ currents (*I*_{Na}) in human pulmonary artery smooth muscle cells (PASM), elicited by depolarizing the cell from a holding potential of -70 mV to a series of test potentials ranging from -80 and +80 mV in 20 mV increments. The inset indicates the summarized *I*-*V* relationship curve for *I*_{Na}. (C) Representative *I*_{Na} at 0 mV from PASM cells before, during and after extracellular application of 1 μM tetrodotoxin.

Figure 12.

Ca^{2+} -activated Cl^- currents (I_{ClCa}) in pulmonary artery smooth muscle cells (PASMC). (A) A representative family of whole-cell I_{ClCa} in PASMC elicited by a series of test potentials from -60 to $+60$ mV in 20 -mV increments (from a holding potential of -70 mV). The cells are superfused with Modified Krebs's solution (MKS) including 1.8 mM Ca^{2+} . (B) Representative traces of I_{ClCa} recorded in PASMC superfused with MKS (Control and Recovery) and Ca^{2+} -free MKS with 10 mM $BaCl_2$ ($0Ca-10Ba$). Replacement of extracellular Ca^{2+} with Ba^{2+} significantly enhanced inward cation current, but abolished the outward Cl^- currents and the inward tail currents, which were carried by Cl^- influx and efflux, respectively, through $ClCa$ channels. (C) The superimposed record of whole-cell I_{ClCa} in PASMC before (Control) and during extracellular application of niflumic acid.

Figure 13.

Gap junction (GJ) and GJ channels in vascular smooth muscle and endothelial cells. The changes in membrane potential (E_m) and $[Ca^{2+}]_{cyt}$ due to Ca^{2+} influx or release in one smooth muscle cell (SMC) can be communicated to an adjacent SMC through GJ channels and to an endothelial cell (EC) through GJ channels in the myoendothelial junction (MEJ). Ca^{2+} and electrical signals can also go through GJ channels between ECs. In addition to Ca^{2+} and other cations/anions, GJ channels also allow small molecules to go through, for example, IP_3 , DAG, and other second messengers in the cytosol. ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; δE_m , changes in E_m (i.e., membrane depolarization, hyperpolarization, or repolarization); ROC, receptor-operated Ca^{2+} channels.

Figure 14.

Formation of gap junction (GJ) channels and topology of the connexin (Cx). (A) A schematic diagram showing how the hemichannels in the membrane of one cell interact with the hemichannels in another cell to form GJ channels. Not all hemichannels form GJ channels; the hemichannels can also function as a non-selective cation/anion channels. (B) The Cx is a membrane protein with four transmembrane domains and cytoplasmic N- and C-termini. There are multiple cysteine residues in the extracellular E1 and E2 segments that make the channel sensitive to the regulation of oxidation and reduction.

Figure 15.

Inhibition of K⁺ channels causes membrane depolarization and causes pulmonary vasoconstriction. (A) Close of K⁺ channels in pulmonary artery smooth muscle cells (PASMC) causes membrane depolarization, which subsequently opens VDCC, enhances Ca²⁺ influx, increases [Ca²⁺]_{cyt}, and induces pulmonary vasoconstriction. Opening of K⁺ channels, on the other hand, causes membrane hyperpolarization (close to the K⁺ equilibrium potential), decreases VDCC activity and causes pulmonary vasodilation. (B) Representative records of whole-cell K⁺ currents (*a*), membrane potential (*E_m*, *b*) and [Ca²⁺]_{cyt} (*c*) in PASMC before (control), during (4-AP) and after (wash) extracellular application of 5 mM 4-aminopyridine (4-AP). A representative record of tension measurement in an isolated pulmonary arterial ring before, during, and after 4-AP treatment is shown in *d*.

Figure 16.

Activity of K^+ channel is involved in apoptotic volume decrease (AVD) and apoptosis. (A) Diagram showing the chronological order of morphological and biochemical changes during apoptotic stimulation. Activation of K^+ channels leads to a loss of intracellular K^+ , which relieves the inhibitory effect of K^+ on cytoplasmic caspases and nucleases, increases caspase-mediated cleavage, and ultimately induces nuclear breakage and apoptosis. Activation of K^+ channels also accelerates AVD, which further facilitates the process leading to apoptosis. (B) Changes in cell morphology before (Control) and during treatment with apoptosis inducers. Exposure of cells to pro-apoptotic triggers activates K^+ (and Cl^-) channels, induces AVD, and ultimately causes DNA fragmentation and nuclear breakage. The image on the right showing DAPI-stained nuclei in pulmonary artery smooth muscle cells treated with staurosporine; the cells undergoing apoptosis (showing significant nuclear breakage) are indicated by arrows. The AVD due to K^+ (Cl^- and H_2O) efflux occurs prior to nuclear condensation and DNA fragmentation.

Figure 17.

Two pathways of apoptosis. When death receptors (DR) are activated (e.g., by Fas ligand), cleavage of procaspase 8 (and/or 10) to active caspase 8 is an important initial step to induce apoptosis. Cytochrome c (Cyt *c*), which can be released from the mitochondria to the cytosol when cells are exposed to UV light or when mitochondrial membrane potential ($\Delta\psi_m$) is depolarized, activates cytoplasmic caspase 9. Active caspases 8 and 9 then activate caspases 3/6/7 and cause DNA fragmentation and nuclear breakage, and eventually cell death. Cytosolic cytochrome *c* also activates K_v channels, induces K^+ loss, decreases cytosolic $[K^+]_{cyt}$, which increases caspase activity and accelerates AVD, and ultimately promote apoptosis (229).

Figure 18.

Cellular mechanisms by which acute hypoxia causes pulmonary vasoconstriction. (A) Multiple pathways are involved in hypoxia-induced increase in $[Ca^{2+}]_{cyt}$ in pulmonary artery smooth muscle cells (PASM). Acute hypoxia can enhance Ca²⁺ influx through receptor-operated Ca²⁺ channels (ROC) by activating phospholipase C (PLC) and diacylglycerol (DAG), through voltage-dependent Ca²⁺ channels (VDCC) by membrane depolarization due to inhibition of K⁺ (K_v and K_{2P}) channels and activation of Ca²⁺-activated Cl⁻ (Cl_{Ca}) channels, and through store-operated Ca²⁺ channels (SOC) by active depletion of Ca²⁺ from the SR/ER. Acute hypoxia can also enhance Ca²⁺ release from the SR/ER, lysosome and mitochondria by activating IP₃ receptors (IP₃R) and ryanodine (RyR) receptors, producing pyridine nucleotides (cyclic adenosine diphosphate-ribose, cADPR and nicotinic acid adenine dinucleotide phosphate, NAADP), and inhibiting electron transportation chain (ETC), respectively. Store depletion may also increase Na⁺ influx through SOC and increases cytosolic $[Na^+]_{cyt}$, which would subsequently stimulate inward Ca²⁺ transportation via the reverse mode of Na⁺/Ca²⁺ exchange (NCX). (B) Representative records of whole-cell K_v currents ($I_{K(V)}$), membrane potential (E_m), and $[Ca^{2+}]_{cyt}$ in PASM before (normoxia), during (hypoxia) and after (recovery) exposure to hypoxia. (C) Acute hypoxia decreases $I_{K(V)}$ only in *KCNK5*-transfected PASM, but not in mesenteric artery smooth muscle cells (MASMC) transfected with *KCNK5* gene.

Figure 19.

Cellular mechanisms by which chronic hypoxia causes pulmonary vascular remodeling and vasoconstriction. Chronic exposure to hypoxia downregulates K_v and K_{2P} channels and upregulates Ca^{2+} -activated Cl^- (Cl_{Ca}) channels in pulmonary artery smooth muscle cells (PASMC). The resultant membrane depolarization opens voltage-dependent Ca^{2+} channels (VDCC) and increases $[Ca^{2+}]_{cyt}$. Chronic hypoxia also upregulates VDCC (α_{1C}), transient receptor potential channel (TRPC1/3/4/6), and Orai2/STIM2 proteins in PASMC. The resultant augmentation of Ca^{2+} influx through these upregulated Ca^{2+} channels further increase $[Ca^{2+}]_{cyt}$ and ultimately causes pulmonary vaso-constriction and vascular remodeling. Downregulation of K^+ channels also contributes to pulmonary vascular remodeling by inhibiting AVD and apoptosis in PASMC.

Figure 20.

Schematic diagram showing the causes of elevated pulmonary vascular resistance in patients with idiopathic pulmonary arterial hypertension (IPAH, A) and the angiographic and histological features of the pulmonary vasculature in IPAH patients (B). The angiogram show significant “loss” of small vessels in the IPAH patient (B, upper panels), which is mainly due to sustained pulmonary vasoconstriction and excessive pulmonary vascular remodeling characterized by medial and intimal hypertrophy, neointimal proliferation, and obliteration of small pulmonary arteries and arterioles (B, lower panels). EVG, elastic Van Gieson staining. The images shown in B are reproduced from Ref. (82).

Figure 21.

Patterns of vascular remodeling (A) and common histological changes in the pulmonary vasculature in patients with pulmonary hypertension (B). (A) The remodeling of a vessel to a larger lumen with the same wall thickness is termed “outward hypertrophic” (or eccentric hypertrophy), since cross-sectional area is increased. Conversely, vessel narrowing with increased wall thickness occurs in chronic hypertension and may be “inward eutrophic” (e.g., smaller lumen with a somewhat thicker wall, but the same cross-sectional area) or “inward hypertrophic” or “concentric hypertrophy” (i.e., smaller lumen with sufficient wall thickening to increase cross-sectional area). A common assumption is that changes in cross-sectional area indicate changes in wall mass (as implied by the terms “hypotrophic” or hypertrophic”). This, of course, is only correct if vessel length is not altered (210). (B) In the lung, pulmonary vascular remodeling consists in concentric hypertrophy, eccentric hypertrophy (associated with adventitial lesion and hypertrophy and extracellular matrix lesions), and obliteration of small vessels due to intimal lesions, plexiform lesions and in situ thrombosis. Concentric hypertrophy, *in situ* thrombosis and obliteration increase pulmonary vascular resistance (PVR) by narrowing the affected arterial lumen, while eccentric hypertrophy contributes to increasing PVR by increasing the wall stiffness or decreasing the vascular wall compliance (which is one of the major causes for reduced distention and recruitment in pulmonary circulation).

Figure 22.

Schematic diagram showing the pathogenic role of dysfunctional K_v channels in the development of pulmonary vascular remodeling in patients with idiopathic pulmonary arterial hypertension (IPAH). Decreased K^+ channel function and expression not only stimulate pulmonary artery smooth muscle cells (PASM C) proliferation by increasing $[Ca^{2+}]_{cyt}$, but also inhibits PASM C apoptosis by attenuating apoptotic volume decrease (AVD) and decreasing cytoplasmic caspase activity. The increased proliferation and inhibited apoptosis in PASM C may play an important role in initiation and/or progression of pulmonary vascular remodeling. VDCC, voltage-dependent Ca^{2+} channels.

Figure 23.

Bone morphogenetic proteins (BMP) enhance whole-cell K_v currents ($I_{K(v)}$) in pulmonary artery smooth muscle cells (PASMC). (A) Schematic diagram depicting the proposed role of BMP-mediated regulation of K_v channel expression and function in human PASMC. There are two types of BMP receptors (BMP-RI and BMP-RII) which dimerize with one another and form a BMP ligand-receptor complex. The activated BMP-RI phosphorylates and activates the receptor-activated Smads (R-Smad) which then form dimerized complexes with Co-Smads and enter the nucleus. The R-Smad/co-Smad interact with DNA in the nucleus and regulate the transcription of various target genes whose primers contain the Smad binding sequence (5'-AGAC-3'). In the nucleus, Smad-1 (a R-Smad) and Smad-4 (a co-Smad) in association with different corepressors appear to be involved in downregulating the expression of Bcl-2, an antiapoptotic protein that blocks the release of cytochrome *c* from the mitochondrial intermembrane space to the cytosol. Bcl-2 also downregulates the mRNA expression and inhibits the function of sarcolemmal K⁺ channels in PASMC. (B) Representative K_v currents (left panels), following 300 ms step depolarization at potentials ranging between -60 and +80 mV from a holding potential of -70 mV, and summarized current-voltage (*I-V*) curves (right panel) in PASMC treated with (BMP-2) or without (Control) 200 nM BMP-2 for 24 h. (C) Representative single-channel K⁺ currents in control and BMP-2-treated PASMC (left panels). Bar graph depicts averaged open-state probability

(NP₀) of single-channel K⁺ currents at +60 mV in contro and BMP-2-treated PASM_C. ** $P < 0.01$ versus Control.

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Figure 24.

Voltage-dependent and -independent regulation of $[Ca^{2+}]_{cyt}$ in pulmonary artery smooth muscle cells (PASMC). Decreased K_v channel activity causes membrane depolarization which subsequently opens voltage-dependent Ca^{2+} channels (VDCC), increases Ca^{2+} influx, and raises $[Ca^{2+}]_{cyt}$. Upon activation of membrane receptors including G protein-coupled receptors (GPCR) and receptor tyrosine kinases (RTK) by ligands, store-operated Ca^{2+} channels (SOC) are activated by store depletion as a result of IP_3 -mediated Ca^{2+} mobilization from the sarcoplasmic reticulum (SR), while receptor-operated Ca^{2+} channels (ROC) are activated by diacylglycerol (DAG). Increased $[Ca^{2+}]_{cyt}$ is a major trigger for PASMC contraction, proliferation and migration.

Figure 25.

Pathogenic role of transient receptor potential channel 6 (TRPC6) in the development of idiopathic pulmonary arterial hypertension (IPAH). (A) Proposed cellular mechanisms involved in excessive increase in $[Ca^{2+}]_{cyt}$ in pulmonary artery smooth muscle cells (PASMC) isolated from patients with IPAH. Upregulated TRPC subunits (e.g., TRPC3/6), due to genetic mutations and/or environmental stimulation, lead to the increased number of receptor-operated (ROC) and store-operated (SOC) Ca^{2+} channels in the plasma membrane, which enhances receptor-operated (ROCE) and store-operated (SOCE) Ca^{2+} entry and increases $[Ca^{2+}]_{cyt}$ in PASMC. Opening of SOC and ROC, potentially formed by TRPC subunits, not only causes Ca^{2+} influx, but also Na^{+} influx. The increased cytoplasmic $[Na^{+}]$ in the close proximity to Na^{+}/Ca^{2+} exchangers (NCX) activates the reverse mode of NCX and promotes inward Ca^{2+} transport. NCX1 is also upregulated in IPAH PASMC in comparison to normal PASMC. (B) and (C) Representative currents (B), elicited by a ramp depolarization from -100 to $+100$ mV, and $[Ca^{2+}]_{cyt}$ (C) before and during application of the membrane permeable diacylglycerol analogue, 1-oleoyl-2-acetyl-sn-glycerol (OAG), in PASMC from normal subjects and IPAH patients.

Table 1
Factors Released from Endothelial Cells

Vasodilators	Vasoconstrictors	Growth modulators	Hemostatic and thrombolytic factors
EDRF:	EDCF:	Promoters:	Pro-coagulant/thrombotic factors:
Nitric oxide (NO)	TXA ₂	Tumor necrosis factor- α	von Willebrand factor
Prostacyclin (PGI ₂)	ET-1	Superoxide radicals	Plasminogen activator inhibitor
Natriuretic peptide			Platelet activator
EDHF:	Other constrictors:	Inhibitors:	<i>Anticoagulant/thrombolytic factors:</i>
NO	Angiotensin II	NO	Tissue factor plasminogen activator
K ⁺	Prostaglandin H ₂	PGI ₂	Tissue factor pathway inhibitor
Epoxyeicosatrienoic acids (EETs)	Superoxide radicals	Protein S	NO
anandamide	Peroxynitrite, ONOO ⁻		PGI ₂
H ₂ O ₂	Hydroxyls, OH ⁻		Protein S

Cytosolic Electrolyte Concentrations Determined by Various Methods in Different Types of Vascular Smooth Muscle Cells

Table 2

Species	SMC Type	[Na ⁺] _{cyt} mM	[K ⁺] _{cyt} mM	[Cl ⁻] _{cyt} mM	Method*
Rat	Portal vein	45	138	86	A
	Aorta	74	110		A
	A7r5 cells	4.4			B
Dog	Mesenteric artery	11			A
	Carotid artery	68	88	70	A
	Aorta	70	129	54	A
Rabbit	Pulmonary artery	15	134	51	A

* Method of determination: A, atomic absorption spectrophotometry and/or flux measurements using radioisotopes; B, use of ion-sensitive fluorescent indicator SFBI. SMC, smooth muscle cell.

Table 3

Expression and Function of VDCC

Sub-type	Voltage sensitivity	α -Subunit gene	Associated subunits	Activators	Inhibitors
L	Strong depolarization	$Ca_v1.1(\alpha_{1S})$	$\alpha_{2\delta}$	BAY K8644	Dihydropyridines
		$Ca_v1.2(\alpha_{1C})$	β		Phenylalkylamines
		$Ca_v1.3(\alpha_{1D})$	$\gamma_{1,2}$		Benzothiazepines
		$Ca_v1.4(\alpha_{1F})$			
T	Weak depolarization	$Ca_v3.1(\alpha_{1G})$	$\alpha_{2\delta}$	Unknown	Mibefradil
		$Ca_v3.2(\alpha_{1H})$	$\beta_{1,3,4}$		Amloride
		$Ca_v3.3(\alpha_{1I})$	Possibly γ		Nickel
					SNX-482 Nickel
R	Strong depolarization	$Ca_v2.3(\alpha_{1E})$	Not known	Unknown	ω -Conotoxin-GVIA
N	Strong depolarization	$Ca_v2.2(\alpha_{1B})$	Not known	Unknown	ω -Agatoxin IVA
P; Q	Strong depolarization	$Ca_v2.1(\alpha_{1A})$	Not known	Unknown	

Table 4

Expression and Function of TRP Channels

Family	Sub-unit	Selectivity P_{Ca}/P_{Na}	Pulmonary expression	Activators	Inhibitors
TRPC	1	Non-selective	SMC, EC	Store depletion, PLC pathway	Gd/La
	2	2.7	SMC, EC	DAG, PLC pathway	
	3	1.6	SMC, EC	Store depletion, DAG, PLC	BTP ₂ /Gd/La/flufenamate
	4	7	SMC, EC	Store depletion? La^{3+} , calmidazolium	Ca^{2+} /2-APB/DIDS/
	5	9.5	SMC, EC	Store depletion? La^{3+} , NO, PLC	2-APB/flufenamate
	6	5	SMC, EC	DAG, PIP ₃ , Ca^{2+} , 20-HETE, PLC	2-APB/La/amiloride
	7	1.9	SMC, EC	Store depletion, DAG, PLC	Ca^{2+} /La/flufenamate
TRPM	1	Non-selective	EC	Translocation?	
	2	~0.3	SMC	ADP-ribose/AA/NAD/H ₂ O ₂ /Ca ²⁺	ADP/clotrimazole
	3	1.6	SMC, EC	Osmotic cell swelling/store depletion?	Mg^{2+} /Gd ²⁺ /La ³⁺
	4	MVC selective	SMC, EC	Ca^{2+} /voltage/PIP ₂ /BTP2/PKC	AMP/ATP/Gd ²⁺ /La ³⁺
	5	MVC selective	—	Ca^{2+} /voltage/PIP ₂ /heat/TIR	Spermine/acid pH (<7.0)
	6	DVC selective	EC	Translocation/acid pH/2-APB	Mg^{2+} /RuR
	7	DVC selective	SMC, EC	ATP/H ⁺ /phosphorylation/PIP ₂	Mg^{2+} /La ³⁺ /PIP ₂
	8	3.3	SMC, EC	Cool/menthol/icilin/pH/PIP ₂	Capsazepine/BCTC/2-APB
	1	3.8	SMC, EC	Heat/vanilloids/anandamide/PIP ₂ /camphor	Capsazepine
	2	3	SMC, EC	Heat/osmotic cell swelling/exocytosis	DAT
TRPV	3	2.6	SMC	Warm/menthol/PUFAs	La
	4	6	SMC, EC	Warm/osmotic cell swelling/5'6'-EET	Gd/La
	5	>100	—	Low[Ca ²⁺]/hyperpolarization/exocytosis	Ca^{2+} /Cd
TRPA	6	>100	—	Store depletion, exocytosis	Ca^{2+} /Cd
	1	0.8	ND	Cold/icilin/isothiocyanates/cannabinoids/bradykinin/DAG/PUFAs	Amiloride
TRPML	1	MVC selective	ND	pH/Ca ²⁺ /proteolytic cleavage	Amiloride/Gd ²⁺ /La ³⁺ /Ni ²⁺ *
	2	—			
	3	—			
TRPP	1	— Non-selective	ND	Ca ²⁺ /TRPP1/EGF/PIP2/actin cytoskeleton	Gd/La/SKF96365/2-APB *

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Family	Sub-unit	Selectivity P_{Ca}/P_{Na}	Pulmonary expression	Activators	Inhibitors
	2	4.3		Ca^{2+} /acid	Flufenamate/Gd/La/Ni [*]
	3	—		Ca^{2+} [*]	Amiloride/Gd/La/Ni [*]

[?] represents controversial observations.

^{*} AA, arachidonic acid; CaM, calmodulin; DAG, diacylglycerol; DAT, 1,3-di-(arylalkyl)thioureas; DVC, divalent cation; EC, pulmonary artery endothelial cells; MVC, monovalent cation; ND, not determined; PIP₂, phosphoinositide-4,5-bisphosphate; PIP₃, phosphatidyl 3,4,5-trisphosphate; PUFAs, poly unsaturated fatty acids; SMC, pulmonary artery smooth muscle cell.

Table 5
Expression and Function of K⁺ Channels in the Pulmonary Vasculature

Family	Subunits	Accessory subunits	Activators	Inhibitors	Antagonists
K _v	K _v 1.1-1.7	K _v β1	Depolarization	Hyperpolarization	4-AP
	K 1.10	K β2	PKA	PKC	Cyt-450 inhibitors
	K 2.1	K β3	Decreased pH _{cyt}	Increased [Ca ²⁺] _{cyt}	(clotrimazole)
	K _v 3.1b/3.3/3.4		Cytochrome c	Rho kinase	Correolide
	K _v 4.1/4.2			Hypoxia	
	K _v 5.1				
BK _{Ca}	K _v 6.1-6.3				
	K _v 9.1/9.3				
	K _v 10.1				
	K _v 11.1-11.3				
	Slo1/KCNMA1	β1	PKG/PKA	PKC	IBTX, ChTX, paxilline, TEA
		β2	NO	ROS	
K _{ATP}		β3	EETs	Decreased pH _{cyt}	(>1 mM)
		β4	CO	c-Src	PDE inhibitors
			AA		
	Kir6.1	SUR2B	PKA/PKG	Increased ATP	
	Kir6.2		CGRP	Ca ²⁺ -activated	Glibenclamide, Tolbutamide
			Decreased ATP	PP2B	
K _{2pd}			Pinacidil	PKC	
	TASK1/2	Not yet identified	Depolarization	Hyperpolarization	Zn ²⁺
	THIK-1		Increased pH _{cyt}	Decreased pH _{ex}	Anandamide
	TRAAK		PKA		
	TREK-1/2				
	TWIK-1/2				
KCNQ	KCNQ1	Not yet identified	Depolarization	Hyperpolarization	

AA, arachidonic acid; CO, carbon monoxide; CGRP, calcitonin gene-related peptide; EETs, epoxyeicosatrienoic acids; NO, nitric oxide; PKA/C/G, protein kinase A/C/G; PDE, phosphodiesterase; PP2B, protein phosphatase 2B.

Table 6
Expression and Function of Na⁺ Channels

Species/Cell	Subunit (gene)	Accessory subunits (gene)	Electrophysiology	Pharmacology
Cultured human PASM	SCN2A-4A, SCN7A-9A, SCN11A	SCN1B, SCN2B	<i>Inactivation</i> $V_{0.5} = -75.5 \pm 3$ mV $\tau_a = 0.485 \pm 0.06$ ms (at 0 mV) $\tau_h = 2.85 \pm 0.41$ ms (at 0 mV)	TTX-sensitive
Cultured human PASM	ND	ND	<i>Inactivation</i> $V_{0.5} = -75.5 \pm 0.8$ mV $K = 10.73$	30% TTX-sensitive
Primary cultured human PASM	ND	ND	<i>Activation</i> $V_{0.5} = -25 \pm 4$ mV $K = 4.2 \pm 0.5$	44% TTX-sensitive
Primary cultured rabbit main PASM	ND	ND	<i>Activation</i> $V_{0.5} = -65$ mV $K = 5.7$	TTX-sensitive

ND, not determined; $V_{0.5}$, half-activation/inactivation potential; K , slope factor for activation/inactivation; τ , time constant (dependency) of activation/inactivation.

Table 7
Expression and Function of Cl⁻ Channels

	Ca ²⁺ -activated (I _{Cl(Ca)})	cGMP-activated (I _{Cl(cGMP,Ca)})	Ca ²⁺ /CaMKII-activated (I _{Cl(CaMKII)})	Volume-sensitive (I _{Cl(swell)})
EC ₅₀ [Ca ²⁺] _{cyt}	160-600 nM	No effect	~430 nM	
EC ₅₀ cGMP	~74 nM	3-6 μM	Not sensitive	
Conductance	1-4 pS		25-30 pS	
Halide permeability	I>Br>Cl	Br>I.Cl. or Cl>I		I>>Br≈Cl>gluconate
Voltage/time dependence	Below 1 μM at resting [Ca ²⁺] _{cyt}	Independent	Voltage-dependent and -independent Ca ²⁺ binding	
Niflumic acid IC ₅₀	2-20 μM	No effect at 100 μM	Not sensitive	
DIDS IC ₅₀	15-250 μM	No effect at 200 μM		
Zn ²⁺ IC ₅₀	Independent	~2-6 μM		
TFP IC ₅₀			5.7 μM	
Protein tyrosine kinase inhibition				Tyrphostin B46(50 μM), Genistein (100 μM)
Gene		<i>Bestrophin-3</i>		