Dysfunctional Voltage-Gated K⁺ Channels in Pulmonary Artery Smooth Muscle Cells of Patients With Primary Pulmonary Hypertension

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Background—Primary pulmonary hypertension (PPH) is a rare disease of unknown cause. Although PPH and secondary pulmonary hypertension (SPH) share many clinical and pathological characteristics, their origins may be disparate. In pulmonary artery smooth muscle cells (PASMCs), the activity of voltage-gated K⁺ (Kᵥ) channels governs membrane potential (Eₘ) and regulates cytosolic free Ca²⁺ concentration ([Ca²⁺]ₘ). A rise in [Ca²⁺]ₘ is a trigger of vasoconstriction and a stimulus of smooth muscle proliferation.

Methods and Results—Fluorescence microscopy and patch clamp techniques were used to measure [Ca²⁺]ₘ, Eₘ, and Kᵥ currents in PASMCs. Mean pulmonary arterial pressures were comparable (46±4 and 53±4 mm Hg; P=0.30) in SPH and PPH patients. However, PPH-PASMCs had a higher resting [Ca²⁺]ₘ than cells from patients with SPH and nonpulmonary hypertension disease. Consistently, PPH-PASMCs had a more depolarized Eₘ than SPH-PASMCs. Furthermore, Kᵥ currents were significantly diminished in PPH-PASMCs. Because of the dysfunctional Kᵥ channels, the response of [Ca²⁺]ₘ to the Kᵥ channel blocker 4-aminopyridine was significantly attenuated in PPH-PASMCs, whereas the response to 60 mmol/L K⁺ was comparable to that in SPH-PASMCs.

Conclusions—These results indicate that Kᵥ channel function in PPH-PASMCs is inhibited compared with SPH-PASMCs. The resulting membrane depolarization and increase in [Ca²⁺]ₘ lead to pulmonary vasoconstriction and PASMC proliferation. Our data suggest that defects in PASMC Kᵥ channels in PPH patients may be a unique mechanism involved in initiating and maintaining pulmonary vasoconstriction and appear to play a role in the pathogenesis of PPH. (Circulation. 1998;98:1400-1406.)

Key Words: potassium ■ electrophysiology ■ pulmonary heart disease

Primary pulmonary hypertension (PPH) is a rare disease with an incidence of 1 to 2 per million per year that is characterized hemodynamically by elevated pulmonary vascular resistance, leading to progressive right heart failure and death. The predominant pathophysiological and pathological features include vasoconstriction, vascular remodeling (medial and intimal proliferation), vascular injury (induced by shear stress, ischemia, and inflammation), and in situ thrombosis.⁴⁻⁻⁻ Impaired endothelium-dependent pulmonary vasorelaxation, increased endothelin-1 synthesis, and imbalanced secretion of vasoactive prostanoids have been implicated in the development of both PPH and secondary pulmonary hypertension (SPH).⁴⁻⁻⁻ However, several lines of evidence indicate that intrinsic abnormalities of pulmonary vascular smooth muscle are present in PPH and may be important in its pathogenesis. Medial hypertrophy, suggesting a stimulus for vasoconstriction, is the earliest and most consistent pathological finding of PPH, and smooth muscle stretch induced by vasoconstriction is a promoter of smooth muscle cell hypertrophy and hyperplasia.⁶ Vascular rings from patients with PPH tend to have more smooth muscle proliferation compared with vessels from patients with SPH.⁷ Additionally, vascular rings from PPH patients are more sensitive to vasoconstrictors than rings from normal subjects,⁸ and patients with PPH tend to be more responsive to acute vasodilator than patients with SPH.⁹ Approximately 26% of PPH patients exhibit significant and sustained reductions in pulmonary artery pressure and vascular resistance in response to Ca²⁺ channel blockers,¹⁰ suggesting that elevated cytoplasmic free Ca²⁺ concentration ([Ca²⁺]ₘ) in pulmonary arterial smooth muscle cells (PASMCs) is involved, at least in part, in the development and maintenance of PPH.

A rise in [Ca²⁺]ₘ in PASMCs is a major trigger for vasoconstriction and an important stimulus for smooth muscle hypertrophy.¹¹⁻⁻⁻ Membrane potential (Eₘ) controls the function of sarcolemmal voltage-gated Ca²⁺ channels and is thus a key determinant of [Ca²⁺]ₘ and pulmonary vascular tone.¹² The resting Eₘ in PASMCs is regulated by the K⁺ channel blocker 4-aminopyridine was significantly attenuated in PPH-PASMCs, whereas the response to 60 mmol/L K⁺ was comparable to that in SPH-PASMCs.
currents through voltage-gated K⁺ (Kᵥ) channels. Inhibition of Kᵥ channels results in cell depolarization, thereby increasing [Ca²⁺]ᵦ and causing pulmonary vasoconstriction. Accordingly, we hypothesized that attenuated Kᵥ channel function, resulting in membrane depolarization and an increase in [Ca²⁺]ᵦ, may play a role in the pathogenesis of PPH. Using patch clamp techniques and quantitative fluorescence microscopy, we compared Kᵥ channel activity, resting Eₘ, and [Ca²⁺]ᵦ regulation in PASMCs obtained from patients with PPH and SPH.

Methods

Subjects
The clinical and hemodynamic characteristics of the 21 subjects from whom lung tissue was obtained are shown in the Table. The diagnosis of PPH was established clinically in 5 patients on the basis of the criteria used in the National Institutes of Health Registry on PPH and confirmed histopathologically. Ten subjects had pulmonary hypertension resulting from known causes (SPH) (Table). Two patients undergoing lobectomy for bronchogenic carcinoma, who had no evidence of pulmonary hypertension by physical examination, ECG, echocardiogram, or pathological examination of resected lung tissue, and 4 patients with obstructive disease, who had normal pulmonary arterial pressures, were the sources of tissue for normotensive control experiments (nonpulmonary hypertension [NPH]).

Preparation and Culture of PASMCs
We used primary cultured PASMCs in this study. Lung tissue, removed from patients in the operating room, was immediately placed in cold (4°C) saline and taken to the laboratory for dissection. Muscular pulmonary arteries were incubated in Hanks’ balanced salt solution (20 minutes) containing 2 mg/mL collagenase (Worthington Biochemical). The adventitia was stripped, and endothelium was removed. The remaining smooth muscle was digested with 2.0 mg/mL collagenase, 0.5 mg/mL elastase, and 1 mg/mL bovine albumin (Sigma Chemical Co) at 37°C to make a cell suspension of PASMCs. The single PASMC was resuspended, plated onto 25-mm coverslips, and incubated in a humidified atmosphere of 5% CO₂ in air at 37°C in 10% fetal bovine serum DMEM for 1 week.

Immunofluorescence Labeling
The PASMCs were stained with the membrane-permeant nucleic acid stain, 4',6-diamidino-2-phenylindole (DAPI, 5 μmol/L), and
the blue fluorescence emitted at 461 nm was used to estimate total cell numbers in the cultures. A specific monoclonal antibody raised against smooth muscle α-actin was used to evaluate cellular purity of cultures, and a secondary antibody conjugated with indocarbocyanine (Cy3) was used to display the fluorescent image (emitted at 570 nm). The cell images were processed by a MetaFluor/MetaMorph Imaging System (University Imagine); the Cy3 fluorescence was colored red and DAPI fluorescence was colored green to display images with red-green overlay.

Measurement of \([\text{Ca}^{2+}]_{\text{cyt}}\)

The \([\text{Ca}^{2+}]_{\text{cyt}}\) in single PASMC was measured by use of fura-2 and quantitative fluorescence microscopy. The fura-2-loaded (3 μmol/L for 30 minutes) cells on coverslips were superfused with the bath solution for 30 minutes at 35°C. Fura-2 fluorescence (510-nm light emission excited by 380- and 340-nm illumination) from PASMCs and background fluorescence were measured with an Olympus IMT2 microscope equipped for epifluorescence microscopy. The fluorescence signals emitted from the cells were collected (at 2 Hz) with a photomultiplier tube and stored for later analysis. When Ca²⁺ is measured with 2 excitation wavelengths for fura-2, \([\text{Ca}^{2+}]_{\text{cyt}}\) is related to the ratio of measured 510-nm fluorescence signals elicited at 380 and 340 nm.

Measurements of K⁺ Currents and \(E_{\text{m}}\)

Whole-cell and single-channel K⁺ currents (\(I_K\)) were recorded with an Axopatch-1D amplifier and pClamp software (Axon Instruments) by use of patch clamp techniques (Figure 1A through 1C). Patch pipettes (2 to 4 MΩ) were fire polished on a microforge. The currents were filtered at 1 to 2 kHz (−3 dB) and digitized at 4 to 6 kHz.

Whole-Cell \(I_K\) Recording

Step-pulse protocols and data acquisition were performed by a TL-1 digital interface (Axon Instruments) coupled to a computer (Figure 1B). Series resistance and whole-cell capacitance were compensated for by adjustment of the internal circuitry of the amplifier. Leakage and capacitance currents were subtracted by use of the P/4 protocol in pClamp software. The normal bath (extracellular) solution contained (mmol/L) KCl 125, ATP 5, EGTA 10, and HEPES 10, pH 7.4, with 1 mol/L NaOH. The patch pipette (intracellular) solution contained (mmol/L) KCl 125, ATP 5, EGTA 10, MgCl₂ 4, and HEPES 10, pH 7.2.

Single-Channel \(I_K\) Measurement

For cell-attached recording (Figure 1A), the extracellular solution was the same as that described for whole-cell current recording. The patch pipette (extracellular) solution contained (mmol/L) KCl 141, KCl 1.2, CaCl₂ 1.8, glucose 10, and HEPES 10, pH 7.4, with 1 mol/L NaOH. The patch pipette (intracellular) solution contained (mmol/L) KCl 125, ATP 5, EGTA 10, MgCl₂ 4, and HEPES 10, pH 7.2.

Membrane Potential Recording

\(E_{\text{m}}\), in single PASMC was measured in current-clamp configuration when the cell was held at no current (\(I=0\)). The extracellular and intracellular solutions were the same as those for whole-cell current recording.

Chemicals

4-Aminopyridine (4-AP, Sigma) and tetraethylammonium (TEA, Chemika Fluka) were directly dissolved in the bath superfusate on the day of use. Charybdotoxin (ChTX, Acurate) and cyclopiazonic acid (CPA, Sigma) were dissolved in water and DMSO, respectively, to make stock solutions of 100 μmol/L and 20 mmol/L; aliquots of the stock solutions were diluted 1:2000 to 1:4000 to make final concentrations of 25 mmol/L and 10 μmol/L. Similar dilution of DMSO alone into the bath solution was used as control and had no effect on K⁺ currents, \(E_{\text{m}}\), and \([\text{Ca}^{2+}]_{\text{cyt}}\).

Statistical Analysis

Data are expressed as mean±SE. Statistical analysis was performed by use of the unpaired Student’s \(t\) test or ANOVA. Differences were considered significant when \(P<0.05\).

Results

Mean pulmonary arterial pressures in SPH (46±4 mm Hg, \(n=9\)) and PPH (53±4 mm Hg, \(n=5\)) patients were comparable (\(P=0.30\)) and were significantly higher than in NPH patients (19±1 mm Hg, \(n=5\), \(P<0.001\)). Other hemodynamic parameters were comparable in SPH and PPH (Table).
Virtually all cells stained by the nuclei acid dye DAPI cross-reacted with the smooth muscle α-actin antibody (Figure 2), indicating that the cultured cells were smooth muscle cells without contamination by fibroblasts and endothelial cells. Additionally, there was no apparent morphological difference between SPH- and PPH-PASMCs (Figure 2).

Resting \( E_m \) and \([Ca^{2+}]_{cyt} \) in SPH- and PPH-PASMCs

Resting \([Ca^{2+}]_{cyt} \) in SPH-PASMCs was not significantly different from that in NPH-PASMCs. However, the resting \([Ca^{2+}]_{cyt} \) in PPH-PASMCs was significantly higher than in NPH- and SPH-PASMCs (\( P<0.05 \)) (Figure 3). The comparable resting \([Ca^{2+}]_{cyt} \) in NPH- and SPH-PASMCs and the significantly different \([Ca^{2+}]_{cyt} \) between SPH- and PPH-PASMCs support the hypothesis that PPH-PASMCs may have a unique defect that does not exist in SPH-PASMCs, despite comparable pulmonary arterial pressures in SPH and PPH patients.

In smooth muscle cells, prolonged membrane depolarization causes sustained elevation of \([Ca^{2+}]_{cyt} \). Consistent with the higher resting \([Ca^{2+}]_{cyt} \), the resting \( E_m \) in PPH-PASMCs was significantly more depolarized than in SPH-PASMCs (Figure 3, inset). \( E_m \) is determined primarily by \( K^+ \) permeability through sarcoplasmic \( K^+ \) channels. Whether the more depolarized \( E_m \) in PPH-PASMCs is due to inhibited \( K^+ \) channels was then examined by comparing \( K^+ \) currents between SPH- and PPH-PASMCs.

Reduced Whole-Cell \( K_V \) Currents in PPH-PASMCs

At least 3 types of \( K^+ \) currents have been described in PASMCs: (1) \( K_V \) currents \([I_{K(V)}] \), (2) \( Ca^{2+} \)-activated \( K^+ \) (\( I_{K(\text{Ca})} \)) currents, and (3) ATP-sensitive \( K^+ \) (\( I_{K(\text{ATP})} \)) currents. Whereas \( K_{Ca} \) and \( K_{ATP} \) currents were minimized with pipette (intracellular) solutions containing 10 mmol/L EGTA and 5 mmol/L ATP, the whole-cell \( I_{K(V)} \) was isolated in SPH-PASMCs (Figure 4A, left). Neither the \( K_{Ca} \) channel blockers TEA (1 mmol/L) and ChTX (25 nmol/L) nor the \( K_{ATP} \) channel blocker glibenclamide (10 μmol/L) affected \( I_{K(V)} \). Similar to rat PASMCs, the \( I_{K(V)} \) in SPH-PASMCs appeared to consist of a transient current and a steady-state current. In PPH-PASMCs, the amplitudes of the currents, measured at the beginning [10 to 50 ms for the transient \( I_{K(V)} \)] and end [250 to 290 ms for the steady-state \( I_{K(V)} \)] of the test pulses (300 ms) (Figure 4A, right), were significantly diminished compared with SPH-PASMCs (Figure 4B). Because the size of the SPH- and PPH-PASMCs appeared similar (Figure 2), the reduced \( I_{K(V)} \) was unlikely to be due to size-related differences in cell capacitance.

Comparison of Single-Channel \( I_K \) in SPH- and PPH-PASMCs

In cell-attached membrane patches of SPH-PASMCs, a large-amplitude \( K_{Ca} \) current and a small-amplitude \( I_{K(V)} \) (slope conductance, 44 to 65 pS; \( n=8 \)) were elicited by depolarization to +90 mV (Figure 5A). The calculated slope conductances of \( K_{Ca} \) currents were 217±8 pS (\( n=17 \)) and 215±7 pS (\( n=9 \)) in SPH- and PPH-PASMCs, respectively. In 44 SPH-PASMC membrane patches tested, the \( K_{Ca} \) current was...
Diminished Response of $[Ca^{2+}]_{cyt}$ to 4-AP in PPH-PASMCs

In excised outside-out patches, 5 mmol/L 4-AP (Figure 6A) had no effect on the large-conductance $K_{Ca}$ current, whereas 1 mmol/L TEA (Figure 6B) significantly inhibited the $K_{Ca}$ current (the steady-state open probability was decreased from 0.65 to 0.23). These results suggest that 4-AP predominantly blocks $K_v$ channels, whereas low doses of TEA selectively block $K_{Ca}$ channels.

Application of 1 mmol/L TEA (Figure 6C) or 25 nmol/L ChTX did not affect $[Ca^{2+}]_{cyt}$ (by $\pm$ 1 nmol/L, n = 10, and $\pm$ 1 nmol/L, n = 17, respectively) in SPH-PASMCs. The $K_{ATP}$ channel blocker glibenclamide (10 mmol/L) also had no effect on $[Ca^{2+}]_{cyt}$ (by $\pm$ 1 nmol/L, n = 17). These results suggest that $K_{Ca}$ and $K_{ATP}$ channels may be relatively inactive under resting conditions because of low $[Ca^{2+}]_{cyt}$ (50 to 100 nmol/L) and a high concentration of intracellular ATP (1 to 3 mmol/L).

The $K_v$ channel blocker 4-AP, however, reversibly increased $[Ca^{2+}]_{cyt}$ in PASMCs from NPH and SPH patients (Figure 7A). This effect was apparently caused by membrane depolarization induced by reduction of $I_{K(V)}$, because a similar effect could be induced by 60 mmol/L K$^+$ (which shifts the $K^+$ equilibrium potential to $-21$ mV) (Figure 7B). In contrast, the 4-AP–induced increase in $[Ca^{2+}]_{cyt}$ was significantly attenuated in PPH-PASMCs (Figure 7A and 7C). The effect of 60 mmol/L K$^+$ on $[Ca^{2+}]_{cyt}$ was similar in cells from SPH and PPH patients (Figure 7B and 7D).

Discussion

The absence of a suitable animal model of PPH and its rarity have hampered progress in clarifying the pathogenesis of PPH. By obtaining pulmonary vascular tissue from patients with both SPH and PPH, we were able to compare and contrast at a cellular level the mechanisms underlying this disease. Our results demonstrate that compared with SPH-PASMCs, PPH-PASMCs have (1) a higher resting $[Ca^{2+}]_{cyt}$ and a more depolarized resting $E_{m}$, (2) an inhibited $I_{K(V)}$, and (3) a diminished response of $[Ca^{2+}]_{cyt}$ to the $K_v$ channel blocker 4-AP. These observations indicate that the $K_v$ channels are dysfunctional PPH-PASMCs. The resultant membrane depolarization and increased $[Ca^{2+}]_{cyt}$ may play a pivotal role in vasoconstriction and possibly vascular proliferation, which are important components of the pathogenesis of PPH.
Dysfunctional PASMC \(K_v\) Channels in the Pathogenesis of PPH

In PASMCs, \(I_{K(V)}\) is composed of the rapidly inactivating \(I_{K(V)}\) [transient \(I_{K(V)}\)], and the slowly inactivating or noninactivating \(I_{K(V)}\) [steady-state \(I_{K(V)}\)]. The transient \(I_{K(V)}\), which resembles A-type \(K^-\) current, is involved mainly in regulating the duration of action potential, whereas the steady-state \(I_{K(V)}\), which resembles slowly inactivating or nonactivating delayed rectifier \(K^-\) current, plays an important role in governing resting \(E_m\). In PASMCs, inhibition of \(I_{K(V)}\) raised \([Ca^{2+}]_{cyt}\) by depolarization of the cell membrane and increased pulmonary arterial pressure, whereas inhibition of \(K_D^\text{ATP}\) currents and \(K_D^\text{ATP}\) currents had no effects on resting \(E_m\), \([Ca^{2+}]_{cyt}\), or pulmonary vascular tone. These results suggest that in PASMCs, \(I_{K(V)}\) is a major determinant of \(E_m\) and \([Ca^{2+}]_{cyt}\) at rest and is an important contributor to the maintenance of basal pulmonary vascular tone.

Weir et al. recently demonstrated that the anorexic agents aminorex and fenfluramine inhibited the 4-AP-sensitive \(I_K\), caused membrane depolarization, and increased pulmonary arterial pressure. Because appetite suppressant use has been implicated in the development of PPH in some patients who take these drugs, it is possible that a predisposition to the development of PPH based on the function of PASMC \(K_v\) channels may exist in susceptible individuals.

Endogenous \(K_v\) channels turn over very rapidly; the half-lives of the channel mRNA and protein are 0.5 and 4 hours, respectively. The short half-life of the \(K_v\) channels suggests that the cells undergo rapid exchange of channel mRNAs. Compared with SPH-PASMCs, the mRNA level of \(K_v1.5\) (a delayed rectifier \(K_v\) channel \(\alpha\) subunit) is significantly attenuated in PPH-PASMCs. The decreased \(K_v1.5\) mRNA expression would reduce the number of the functional \(K_v\) channels and decrease \(K_v\) current availability. Thus, 1 mechanism involved in the attenuated \(I_{K(V)}\) in PPH-PASMCs is inhibited gene transcription and/or reduced mRNA stability of \(K_v\) channels.

\([Ca^{2+}]_{cyt}\), Pulmonary Vasoconstriction, and Vascular Remodeling

In vascular smooth muscle cells, \([Ca^{2+}]_{cyt}\) can be increased by \(Ca^{2+}\) influx through \(Ca^{2+}\) channels and \(Ca^{2+}\) release from intracellular stores. Because of the voltage dependence of the sarcolemmal \(Ca^{2+}\) channels, membrane depolarization is an important cause of elevated \([Ca^{2+}]_{cyt}\) in PASMCs. The voltage window for sustained elevation of \([Ca^{2+}]_{cyt}\) through smooth muscle voltage-gated \(Ca^{2+}\) channels ranges from -40 to -15 mV. Therefore, the more depolarized PASMCs in PPH patients result in an increased \(Ca^{2+}\) influx and elevated \([Ca^{2+}]_{cyt}\).

The ratio of cytosolic free \([Ca^{2+}]\) to the intracellularly stored \([Ca^{2+}]\) in the sarcoplasmic reticulum is about 1:10 000 to 50 000. The resting \([Ca^{2+}]_{cyt}\) in PPH-PASMCs is \(~\)23% higher than in SPH-PASMCs, which would be expected to result in a significant increase in \([Ca^{2+}]_{cyt}\). Agonist-induced vasoconstriction is triggered by an initial release of \(Ca^{2+}\) from sarcoplasmic reticulum. The higher \([Ca^{2+}]_{cyt}\) may thus be responsible for the augmented agonist-mediated pulmonary vasoconstriction in PPH patients.

A rise in cytosolic \([Ca^{2+}]\), in addition to triggering cell contraction, can rapidly (within 50 to 300 ms) increase nuclear \([Ca^{2+}]\) and promote cell proliferation by moving quiescent cells into the cell cycle and by propelling the proliferating cells through mitosis. Thus, increased \([Ca^{2+}]_{cyt}\) may also play a pivotal role in the hypertrophy of small pulmonary arteries and muscularization of pulmonary arterioles, which are characteristic of PPH.

Possible Origin of PPH: Involvement of Dysfunctional \(K_v\) Channels

A variety of endothelium-derived vasoactive substances, such as nitric oxide, endothelium-derived hyperpolarizing factor (epoxides), prostacyclin, and endothelin, exert their effects in part through alteration of \(ion\) channel function in PASMCs. Thus, an impairment of endothelium-dependent pulmonary relaxation, an imbalance in the ratio of vasoconstrictors and vasodilators, and a dysfunctional \(K_v\) channel in PASMCs may contribute to the development or progression of PPH. We postulate that the early stages of PPH are characterized by pulmonary vasoconstriction resulting from dysfunctional \(K_v\) channels (Figure 8), which lead to \(E_m\) depolarization and an increase in \([Ca^{2+}]_{cyt}\) in PASMCs. Subsequently, impaired endothelium-dependent vasodilation and elevated \([Ca^{2+}]_{cyt}\) potentiate vasoconstriction and eventually lead to vascular remodeling. Altered secretion of endothelium-derived constricting and relaxing factors further contributes to vasoconstriction and vascular wall thickening. Ultimately, dynamic vasoconstriction is replaced by extensive vascular remodeling.
18. Fleischmann BK, Murray RK, Kotlikoff MI. Voltage window for susceptibility to calcium-induced Ca²⁺ release from intracellular Ca²⁺ stores (mainly sarcoplasmic reticulum [SR]) raise [Ca²⁺]SR, which triggers pulmonary vasconstriction. Rise in [Ca²⁺]SR would also increase nuclear Ca²⁺ concentration ([Ca²⁺]N) and stimulate cell proliferation, which causes pulmonary vascular remodeling. Endothelin-derived relaxing factors (EDRF) may participate in regulating Ca²⁺ release through activation of K⁺ channels. Increased Ca²⁺ influx through voltage-gated Ca²⁺ channels in PASMCs. (+) indicates increase (or enhance); (−), decrease (or inhibit).

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References
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